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PHYSIOLOGICAL REVIEWS

VOL. 16 JANUARY, 1936 No. 1

THE SYNTHESIS OF PROTEINS IN VIVO

ROBERT SAXELBY ALCOCK

Cambridge, England

Anabolism and catabolism. The study of metabolism, as pursued up to the present, is the study of catabolism. The contrast between the detailed chemistry of catabolism on the one hand, and the vague generalisations with regard to anabolic processes on the other, stands out keenly, and in no group of substances is this so clearly seen as with the proteins. With the exception of the mode of action of pepsin, and to some extent, the mechanism of deamination, it may be said that the study of protein catabolism has met with considerable success at all points; the hydrolysis to the amino-acids; the identification of these; the studies on proteolytic enzymes; ketolysis and ketogenesis; urea synthesis:—all these processes are more or less understood, and it is possible to trace in some detail, in normal conditions, a high percentage of protein nitrogen from one end of its course in the body to the other.

What is known of the corresponding anabolic processes? Nitrogen, ingested in the form of protein, is laid down as protein in the tissues. The amino-acids are generally assumed to be the highest common factor in this change, and the anabolic process is regarded as the synthesis of body protein from the amino-acids traversing the walls of the intestinal canal.

The only observations calculated to throw any light on this synthesis are those of Blackwood (1932) following the earlier work of Cary (1920, 1926); namely, that in the blood stream afferent to a site of protein synthesis, the amino-acids are in higher concentration than in the efferent.

Catabolic processes are readily stimulated, initiated and controlled. Control of the anabolic processes is only through hormones of unknown action; protein synthesis can be stimulated by the administration of the anterior pituitary growth hormone, but this has, thus far, thrown no

light whatever on the methods of the synthesis which it has the power to initiate.

The catabolic processes may be conducted *in vitro*, and in some cases, imitated by models. Protein hydrolysis may be effected by enzymes *in vitro*, or by acid or alkali. Not so the corresponding anabolic processes. The tissue culture, the only case of protein synthesis under any measure of control, carries out its work as secretly as the whole animal, and has so far yielded no information.

The case of plastein formation, a reputed enzymatic synthesis of protein, will be discussed later. Suffice it to say here that, if there is really a synthesis, it can best be described as a polymerisation, and the product probably has little relationship with the proteins from which it ultimately derived.

In the absence of evidence of any sort, there has been a tacit acceptance of what must be considered as the logical theory of protein synthesis. Protein analysis means protein hydrolysis. Protein synthesis, therefore, becomes a condensation process. This view has a wide, almost universal acceptance, yet the actual evidence in its favour is nil. The process of condensation has never been demonstrated; no enzymes are known which can bring it about. The condensation involved in plastein formation is probably of a different type. It is thus clear that if actual evidence can be brought against this view, it will be readily abandoned.

The difficulty of building a theory of protein synthesis lies mainly in the impossibility of finding an "Angriffspunkt." No polemic with the supporters of the old theory is possible; it admits of no support. It is always easier to build up a new theory on the ruins of an older; the old theory in this case has no foundation, and its ruins do not lend themselves to building. It becomes necessary, therefore, to start *de novo*. The demolition of the old idea is essential, but the new building must be carried on side by side with that process.

Protein origins. Morphological characteristics may be studied most conveniently by reference to their evolutionary origins. The method has not been applied extensively to chemical make-up, but it is conceivable that, in cases where the other investigation methods fail, this might profitably be used. If ontogeny recapitulates phylogeny, in cases where the ontogeny is difficult to follow, a study of the evolutionary development might provide compensation.

In the case of protein synthesis, such a study can be little more than a series of surmises, the process of its evolution being probably the earliest

in the history of life. In considering the early forms of life, however, it is almost inevitable to postulate some form of living molecule; a colloidal micella, comprising a foundation, with specific groups at various foci, each performing one of the functions recognised as characteristic of life. There would be groups maintaining a type of autoxidation, and others capable of using the energy arising from this for the purpose of synthesis. The greatest probability is that the foundation would be of carbon-nitrogen, the actual attachment being of the biuret type, and the specific functional groups, the amino-acid rings and chains. A tentative exemplification of this conception would suggest for the autoxidation control; SH groups, and possibly pyrrole, for growth (reproduction); the basic amino acids, in particular, histidine and arginine. In the absence of a boundary wall, which would preclude the use of crystalloids, the hormone functions would also be carried out by such specific groups, in much the same way as the hematin attached to globin enables it to carry oxygen.

Such an organism might be described as an autotrophic virus. The known viruses are recognised, not by their presence, but by their activities. If a virus were to exist, which exerted no characteristic effect, pathogenesis, bacteriophage, and so on, it would, in the nature of things, escape notice. And it is, therefore, possible that these primitive forms are still in existence, but have thus far escaped detection.

This primitive living molecule would represent a gradually complicating system. As the evolution proceeded, new functions would develop, until, at a certain stage, a more highly organised system would give rise to the first cell. The transition would be conditioned by the saturation of the single molecule organism—the higher organisation would call for a greater mobility, and better intercommunication than would be possible between the active foci on a single molecule. The molecule, at this saturation point, would be carried over into the new type of organism, which would, therefore, inherit all the old characteristics, together with appropriate modifications. It is also to be expected that there would also be a development along the old line—an extension of the non-cell idea, and it may be that such an extension is to be seen in the modern pathogenic and bacteriophagic viruses.

The protein molecule at this transition point thus becomes the parent of all true proteins, and represents the basis of parenchyma proteins. It might be styled the "Urprotein," to indicate its fundamental position. This urprotein would possess certain characteristics, and these we may trace in the properties common to all proteins, in contradistinction to the

specific functional characteristics. One of these properties will be amino-acid constitution.

If it is allowed that the simpler aliphatic amino acids are largely equivalent, and that, say, the exchange by a protein of a percentage of glycine for alanine is likely to be of no great moment, there exists a very real uniformity among proteins, which may be taken as providing a clue as to the composition of the urprotein.

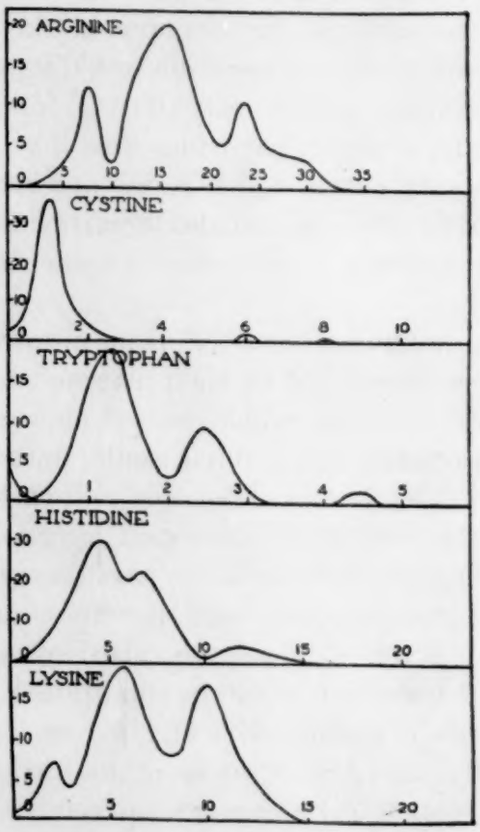

Fig. 1. Abscissa for tryptophan curve; percentage of tryptophan in proteins.
Abscissa for other curves; percentage of the amino-acid in proteins, expressed as nitrogen.
Ordinates: numbers of individual proteins.

This may be seen in the figures, in which the percentage of an amino-acid in proteins is plotted against the frequency of occurrence; the curves show pronounced norms. The peak percentages would thus be the composition of the urprotein. The other peaks will represent some functional characteristics of the few proteins involved; in the case of those on the left of the main peak, there would be an actual elimination of the amino-acid already present; those to the right, an addition.

A similar view, but rather more restricted in scope, and resting directly on the foundation of experimental results, is put forward by Block (1933, 1934). He suggests that there is an Anlage of the basic amino-acids in all proteins. He demonstrates the constancy of this for both birds and mammals, and stresses "the primary importance of the basic amino-acids in the genetic and embryological development of the tissue protein as it exists in protoplasm." It will be seen from the figures here shown, however, that the curves for tryptophan and cystine are more clear-cut in their significance than those for arginine and lysine. These figures were compiled from a variety of sources, and represent all known types of proteins.

According to the view propounded above, the synthesis of protein would involve two processes; the development of the urprotein, and the subsequent differentiation of this to provide the particular substances characteristic of the tissues; myosin, keratin, and so on.

The building up of the protein must, historically, have involved the development of more complex groupings from simpler ones; the rings and special groups, such as the guanidine radical, would be developed within the actual protein molecule, from the primitive groups of the earlier stages. Thus if the same process is still being followed, the first stage in protein synthesis is the formation of some simple skeleton molecule from which specific groups characteristic of the amino-acids develop later, according to some general rule, and giving rise to the urprotein. It is hoped to show that these processes probably are actually involved in protein synthesis in the animal organism.

The story has been built largely on a series of analogies, rather than surmises, and follows closely the use of the cell in forming the multicellular organism. Alone, it contributes little to a theory of protein synthesis, but if the actual experimental evidence would suggest a similar method of synthesis, its support becomes of value.

The review deals exclusively with the synthesis of proteins in the animal organism—little enough is known of that, but even less seems to have been discovered regarding the corresponding process in the plant, but it may be said here that, on *a priori* argument, it must follow some such course as that outlined above. Some time is occupied in the following pages in showing that the animal starts with something simpler than the amino-acid in starting to build up its protein; there is no doubt that this is the case with the plant, and it is highly improbable that the first step in the synthesis should be that of building up the whole series of amino-acids individually. The greatest likelihood is the polymerisation

of some simple synthetic substance, such as a condensation product of formaldehyde with ammonia or nitrous acid. The homogeneous molecule so formed would undergo a process of internal differentiation during which the amino-acids would be formed.

And with a fundamental process of this kind, it is justifiable to suppose that what applies to one type of protoplasm applies to another, and that therefore, in the animal, the amino acids are broken down to this simple substance, or something analogous, whose polymerisation provides the basis for the parenchyma and tissue proteins.

ANABOLISM OF PROTEIN. *Nitrogen retention.* The qualitative aspects of nitrogen retention have been thoroughly studied in connection with the biological values of proteins, which is of commercial importance. And the first and most obvious fact emerging from these studies is that, per gram of nitrogen, different proteins have widely differing values, as measured by the growing animal's ability to convert them into tissue protein. The values range from keratin, silk fibroin, elastin, etc., which do not yield to enzymatic hydrolysis, through gelatin (which fails in both regards), zein, gliadin, which lack in certain amino-acids, to proteins such as myosin and albumin, which are complete in all respects, and readily digested. The failure of the enzymes of the intestine to hydrolyse certain proteins is without significance in this regard. The synthetic process utilises amino acids entering the portal system, and in these cases, there are none.

The failure of some proteins, once hydrolysed, to support growth, is found to be due almost entirely to some deficiency in amino-acid make-up. Such proteins are found to be lacking in one or more of the essential amino acids; tryptophan, cystine, histidine, or lysine. Failure to grow carries with it failure to lay down body protein, which in turn, implies a failure to produce the normal quantities of the essential amino-acids in question. It might, therefore, be supposed, and is, in fact, usually so assumed, that the failure to grow imposed by an amino-acid deficiency is due to the inability of the animal to make the amino-acid in question. This is manifestly true in the last analysis for the sulphur-containing amino-acids, but there is more doubt attaching to the others.

The alternative hypothesis: that the association of the lack of the amino-acid in the diet, and the failure to lay it down in the tissues, is fortuitous, will be discussed in the section on the significance of the indispensability of certain amino-acids.

Beyond this, the general nitrogen-balance experiments indicate that the deposition of protein is governed by factors outside the control of the

experimenter. The anterior pituitary growth hormone, and the vitamin growth-factors are exceptions, but their mode of action, and the part they play in conditioning protein synthesis, is unknown. It is now generally assumed that the amino-acids are the only possible source of nitrogen for protein building in the animal. Mitchell and Hamilton (1929, p. 571), in summing up the conclusions drawn from a multitude of experimental results, conclude that, in animals other than ruminants, whose bacterial symbiotes place them in a unique position, the nitrogen of fed ammonium salts is not available for protein synthesis. The nitrogen retention which has been observed, is not accompanied by a retention of sulphur, and is, therefore, probably not held in the form of protein (Gessler, 1920). In the case of urea, there is no retention. It may therefore be stated that nitrogen which is to be built up into protein by the animal must reach the tissues in the form of amino-acids, either free, or combined.

Mention has already been made of the work of Cary (1920 and 1926) and Blackwood (1932) showing that, for rapid protein synthesis, there is a disappearance of blood amino-acids. It is seen in the lactating cow, where the concentration of amino-acids in the vessels afferent to the mammary glands is greater than in the efferent. The values were controlled against the effect of water absorption by the gland, by means of hematocrit determinations. These results imply, of course, that the nitrogen source for casein is the free blood amino nitrogen. It seems likely that the other milk proteins are taken directly from the blood. But it cannot be said that these observations prove in any way that the amino-acids found in the casein are the amino-acids which disappear from the blood. There is no method available for marking amino-acids. In fact this evidence does little more than establish what could have been argued out from first principles. The nitrogen in the casein must be brought from somewhere; the only vehicle is the blood. If the blood proteins are the actual source for casein nitrogen as well, the difference in constitution implies a fairly complete hydrolysis before the synthesis. It is much easier to imagine that the free amino-acids are used. Thus, though these experiments give results which are in accordance with the conception of protein-building by a piecing together of the constituent amino-acids, they do not in any sense prove it. They will, on the other hand, fit in with the theory here propounded: the idea that the building involves a preliminary breakdown of the amino acids. In this sense, the blood amino-acids represent, not the amino-acid vehicle, but the nitrogen vehicle, for the new synthetic process.

Anabolism in embryos and tissue cultures. It can only be said that the work on the nitrogenous metabolism of embryos has shed no light upon the mode of protein synthesis. In the developing egg there is a complete reconstruction of the proteins and certain changes in the free amino acid concentrations indicate that the nitrogen is carried from one place to another in this form (Needham, 1931). There is at no stage a high concentration of the likely intermediates—polypeptides, etc., a condition also observed in growing tissues at a later stage. But the actual synthesis is conducted with the same inscrutable efficiency that characterises the synthesis occurring in the grown animal, and actually nothing can at present be said as to the course of the reactions. Tissue cultures have been even less investigated from this point of view, and contribute practically nothing. It was found (Willmer and Kendal, 1932) that growth in the embryonic heart of the chicken was accelerated by some polypeptide, whose nature was not established. It is doubtful whether this can be considered as being of significance in this connection.

Plastein formation. Most of the information with regard to protein breakdown has been obtained from experiments carried out *in vitro*. It might be hoped that the elucidation of the synthetic method would yield to the same techniques. The work which does appear to demonstrate a synthesis of protein is that on the "plastein" forming powers of the proteolytic enzymes. The whole subject has been exhaustively reviewed by Wasteneys and Borsook (1930) who have, indeed, made the major contribution to our knowledge of it.

Placed in contact with the products of peptic digestion, in high concentration, and at pH 4.0, pepsin, and to a less extent, trypsin, will bring about the formation of a substance which has been called "plastein." This substance is a protein. At pH 1.7, it is digested by pepsin, and there is an increase in both the COOH and NH_2 concentrations, corresponding with the decrease which accompanied the "synthesis." The material is markedly less soluble than the peptone from which it is made, and these two criteria have been widely accepted as evidence of its complexity.

Folley (1933) questions the validity of this conclusion, and claims for plastein, which he says is itself a mixture, a molecular weight of less than 1000. The determination was carried out by the ultracentrifuge method in Professor Svedberg's laboratory, on a sample supplied by Folley. The evidence given would have had more significance had this maximum been bigger, but as it is, it limits the plastein molecule to seven or eight amino-acid molecules, which, for the material described by Wasteneys

and Borsook, seems highly unlikely. Nevertheless, the proofs of complexity seem insufficient. There are, in fact, two possibilities; either there has been a polymerisation of the peptone molecules, producing a compound of higher molecular weight and lower solubility (precipitable by tri-chloracetic acid), or else there has been an extensive anhydride production, causing at once a diminution in the amino- and carboxyl-concentrations, and a decrease in the solubility. The association of low molecular weight and insolubility is not necessarily paradoxical—the amino acids are markedly less soluble than the lower peptides. If the process is indeed one of condensation within the molecule, then the observations are in no way germane to the question of the mode of protein synthesis. If there has been an increase in complexity, it must be admitted that the product of the polymerisation is not the protein from which the peptones were originally derived, and it is probably safer to describe the reaction as peptone polymerisation, than as a synthesis of protein.

Blagowestschenski and Jeremejew (1934) have repeated the work of Wasteneys and Borsook, and come to substantially the same conclusions. But they are content with the same criteria of synthesis, and produce no actual proof of increase in molecular size. One of the witnesses of synthesis is the digestibility by pepsin at pH 1.7. This would appear to beg the question. If the enzyme can build plastein, it should be able to break it down again, quite irrespective of the nature of the reaction involved. The question will become clearer when it is known what are the substrates of pepsin.

In their review, Wasteneys and Borsook outline the theoretical arguments leading them to expect a synthesis of protein by pepsin. If pepsin can bring about the one reaction, it should also be able to bring about the reverse. The fact that the reversal is effected at a wholly different pH (the isoelectric pH, not that of the fully ionised molecule) suggests that it is, in effect, no simple reversal but a totally different reaction. But if, on theoretical grounds, it is expected that pepsin will also synthesise, the occurrence of plastein loses any significance in respect to the synthesis of proteins in the living cell, failing an actual proof that the conditions governing the reaction do obtain there, and that pepsin itself occurs there. The synthesis, as it stands, and if it is indeed a synthesis, is a consequence of the nature of pepsin, and nothing more.

There is one isolated observation of the synthesis of a protein, or rather of a protamine, from amino-acids (Taylor, 1907). Whether the protamines come into the same category as the proteins seems very doubt-

ful—they conform to none of the usual rules of structure—as regards amino-acid constitution, for instance. The other reported syntheses all involve the reversal of reactions which themselves are unknown. The observations of Abderhalden (1916), that the tissues themselves can be made to resynthesise proteins from the products of autolysis, and that these syntheses are tissue-specific, are significant. They have never been repeated, however, and as in the case of plastein formation, some doubt attaches to the nature and complexity of the synthetic product formed.

In fact, it may fairly be stated that, so far, no mechanism has been demonstrated which might conceivably bring about the synthesis of proteins in the tissues, starting from the amino acids. With regard to this fundamental reaction we have no information whatever and certainly the *in vitro* experiments on plastein formation would seem to have no bearing on it.

The non-essential amino-acids. If the proteins are built up by piecing together the molecule from small fragments, like a stained-glass window, then one of the first essentials for the process is an ample supply of all the various constituents. So if it can be shown that protein synthesis in the tissues can occur in the absence of one or more of the amino-acids it seems likely that either they must be to some extent interchangeable, or that the essential basis for protein building is something simpler than the representative amino-acid mixture usually postulated.

Now protein synthesis, and particularly the synthesis of the tissue proteins, is a relatively slow process, and proceeds diffusely throughout the organism. Any attempt to interfere with it tends to stop it altogether. It seems inevitable, therefore, that the experimental material should be the whole animal, rather than any individual part. Supposing that the supply of one of the amino acids is interrupted; what evidence is there that it will be missing from the blood amino-acid complex in time? The instance which presents itself is glycine, and the evidence derived from the formation of hippuric acid, on the administration of benzoic acid. This would suggest that, even in the absence of dietary glycine, the blood can be kept supplied with the amino-acid, for the formation of hippuric acid will go on indefinitely whether glycine is included in the diet or not. But it appears likely that this example is exceptional, and cannot be regarded in any way as representative of the state of affairs existing for other amino-acids. The glycine of hippuric acid is probably a secondary product, the reaction involving the formation of benzoyl ornithine, or ornithuric acid, which is subsequently broken down to hippuric acid. As glycine in this case is not produced

free, but only in combination with benzoic acid, the reaction could not be used for compensating a shortage in the blood-stream.

It is necessary to picture the state of affairs in an animal living on a diet lacking in some one amino-acid; say glutamic acid. During the shortage of glutamic acid the animal continues to build up proteins (and glutathione); continues, in fact, to lay down glutamic acid, and it is possible to maintain an animal in this way so long that it may lose appreciable quantities of the amino-acid in its shed skin and hair. Where does this glutamic acid come from? There are two possibilities. It may be synthesised in some special organ, say the liver, or it may on the other hand be produced locally at the site of protein synthesis. If the former, then it would seem that some mechanism exists, compensating the shortage of glutamic acid by a specific synthesis. The same must apply for all other dispensable amino acids. The possibility, which implies an extremely complex mechanism, which is only called into play under the most abnormal conditions, must be dismissed as highly unlikely.

If, on the other hand, the amino acid is produced where the protein is synthesised, it may be made either before, during, or after, the actual synthesis. To suggest that it is made before, is to imply for all tissues the complicated machinery which has been dismissed as improbable for one. It is certainly unlikely that a tissue, finding itself short of one amino-acid, will be able to make good the deficiency in this simple manner.

All that is left is to assume that the amino-acid groups appear for the first time in the proteins they ultimately comprise, and the composition of the feeding medium is, with certain reservations, immaterial. It is possible that the nitrogen must be in the form of amino groups —the nitrogen retention reported on ammonium salts is usually discredited. The ability of the organism to synthesise proteins while not being supplied with certain of the amino-acids, would seem to suggest, then, that this synthesis is much more far-reaching than is usually supposed; the amino acids are disintegrated to some simple groups, which are then built together—by an enzyme system which might in itself be quite simple—to form a homogeneous molecule. Out of this, heterogeneity, providing the specific rings and chains of the amino-acids, would develop later.

The essential amino-acids. Certain of the amino-acids are not dispensable in this way. Deprival results in a syndrome resembling that following a vitamin lack; in particular, there is a failure to grow, or even

a definite loss of weight. It has often been assumed that this must represent a limitation to protein synthesis imposed by the amino acid lack. Taking the case of tryptophan, it is clear that the animal's power to synthesise this amino-acid is limited. If it is indeed wholly unable to make it, it will, of course, be unable to make the proteins containing it, and practically all proteins do. If, then, it can be shown that failure to grow on a tryptophan deficient diet is due, either wholly or in part, to the resulting inability to synthesise protein, it will seem likely that the "jig-saw" synthesis of protein is an actual fact. If, on the other hand, it can be shown that tryptophan has some other function which will account for its indispensability, and that the observed losses in weight are due to this; that the failure of the tissues to lay down tryptophan in the tissue proteins is only fortuitously associated with its absence from the diet, then this "jig-saw" synthesis will become less probable. If further, it proves possible to demonstrate that protein synthesis can take place in the absence of dietary tryptophan, it will then be almost certain that the free tryptophan of the blood does not enter directly into the synthesis.

The position is clearer than with the non-essential amino-acids, for in these cases, there is the knowledge that the organism finds no difficulty in synthesising the substance. With the essential amino-acids, this difficulty does exist. If the tryptophan required for protein synthesis runs short, it clearly cannot be supplied by tryptophan synthesised elsewhere; such a synthesis is outside the animal's powers. So the issue turns on the ability or otherwise of the animal to make proteins in the absence of dietary tryptophan, and on the significance of the indispensability of this, and of similar amino-acids.

The functions of the individual amino-acids have received little study, but it becomes clear, in reviewing the existing evidence, that their importance has been under-estimated. The difficulty of studying them, and the fact that they are relatively free from pathological associations, accounts for the lack of study, but if their protein connections are to be understood, it must be known whether their sole significance lies in their use as protein building-stones, or whether they have specific and individual functions as well.

They should, for these purposes, be considered separately, but the experimental studies thus far carried out are few. Glycine appears in the machinery used in the detoxication of benzoic acid, though possibly only secondarily. Glutamic acid provides glutamine, which the human organism uses for the detoxication of phenyl acetic acid. It also,

together with glucine and cystine, goes to form glutathione, and has, in addition, been suggested as a pyrrole precursor. Arginine and histidine are clear examples of amino-acids with individual functions; the former is at the basis of the vertebrate urea synthesising mechanism, the latter is indispensable, and is associated both with blood formation and purine metabolism, while both together are probably of importance in connection with nuclei. Tyrosine and/or phenyl alanine form the basis of the melanin reaction and are probably the precursors of adrenaline and thyroxine. Tyrosine is not essential, though phenyl alanine may be (Alcock, 1934a).

Tryptophan has some special function. It has been suggested that this is that of providing the pyrrole group for hematin, but it has been possible to show that rats can continue to make pyrrole in the absence of dietary tryptophan. The syndrome resulting from its lack has yielded little information. Rats so deprived fail to grow, and in general, show gradual losses in weight; the fat stores are depleted; the hair tends to fall out, and the skin to harden. The experiments of Berg and Rose (1929) have a direct bearing on the subject of protein synthesis. These observers showed that the demand for tryptophan is immediate; that feeding large doses at relatively long intervals is not equivalent to feeding smaller doses at shorter intervals. If the rat is starved of tryptophan for more than 24 hours, it shows the effects. This would point to some function associated with the more fluid aspects of the animal economy. The effects of protein synthesis failure could hardly make themselves felt so soon.

The other chapters in the tryptophan story treat of its breakdown to kynurenic acid through kynurenin. This seems to take place in cases of a sudden flood of tryptophan, and the phenomenon exhibits a kind of threshold effect. Kynurenin and kynurenic acid have not yet been shown to possess any physiological function, and, although it would be premature to make any dogmatic statement, one would tend to view the mechanism as being there for the detoxication of excess tryptophan, so to speak; an arrangement for getting rid of a surplus from the systemic circulation, which might be harmful to the organism. The association between tryptophan, kynurenin, and urochrome will acquire significance only when the functions of one or more of these substances have been established.

Allusion has already been made to the hematopoietic function of tryptophan, and the evidence on this point is briefly: that tryptophan, when injected into an animal rendered anemic, either by hemorrhage, or

by the injection of phenyl hydrazine, will accelerate the return to the normal blood figures (Hirazawa, 1921; Iwakura, Otani and Taniguchi, 1933). It has been further stated that normal animals respond in a similar manner, by showing hyperemia as a result of tryptophan, or tryptophan and histidine injections (Fontés and Thivolle, 1930). These observers assume that the action is simply that of supplying a constituent necessary for the production of hematin; that, in fact, the concentration of blood tryptophan is a limiting factor in hemoglobin production. They apply the same argument to histidine and globin, thus presenting an anomalous case of one reaction being limited simultaneously by two factors. Now it is quite clear that if hematin is really produced from tryptophan, tryptophan deficient animals will become anemic. The French workers actually found that this was so. Work in this laboratory, on the other hand, contradicts it, and also shows that, even in the absence of dietary tryptophan, recovery from anemia is possible (Alcock, 1933). It has been shown (Matsuoka and Nakao, 1931) that the injection of 2 methyl tryptophan exerts a similar effect to tryptophan itself in curing anemia, while not being able to replace it in the diet.

It is clear, therefore, that while tryptophan has some definite function, necessary to normal health and growth, and while it does exert a hematopoietic effect, these are not one and the same. The nature of this function is still a mystery.

Proteins and the essential amino-acids. It has been stated that there is a failure to grow in the absence of dietary tryptophan; there is also a specific failure in certain protein-forming tissues; in particular, the skin and hair are undernourished. But it has not been shown that this deficiency in protein synthetic powers is complete. In the first place, it is possible to keep rats alive for six months without any tryptophan in their food. Is it to be supposed that, in this time, they synthesise no protein? They make some skin and hair. They make intestinal secretions containing proteins. Furthermore, they make blood in considerable quantities, and it has been shown (Alcock, 1933) that they are capable of actually recovering from anemia on a deficient diet. And while they may not make use of tryptophan for building hematin, they probably incorporate it in the globin.

The occasional rats which lose no weight for protracted periods on a tryptophan deficient diet, quite clearly lay down protein tryptophan during this time; reconstruction in the body may not be extensive, but it certainly does exist. An example of this was arrived at by accident. An animal placed on a tryptophan-deficient diet was found to be preg-

nant. She maintained her weight at a steady level for a fortnight, and gave birth to six young rats, dead, and undersized. But these young rats had been built up from the mother's tissues and foodstuffs, during her period of tryptophan starvation. As there was no increase in weight, it might be thought that the tryptophan in their tissues was that coming from the compensating dissolution of tissue in the mother. This would argue an unbelievable economy of tryptophan, whereby all that liberated by autolysis was transferred quantitatively to the young. It is, in short, clear in this case, as is most probable in the others, that protein synthesis was proceeding.

Further evidence of such synthesis is presented in a paper at present in the press. It is shown that, over short periods, animals on a tryptophan-deficient diet, injected with the anterior pituitary growth hormone, show definite increases in weight, which in all probability represents growth and protein synthesis. The failure of animals to put on weight in tryptophan deficiency is definitely to be referred to the interference with the "essential" function, and there is no difficulty in believing that protein synthesis would go on unimpaired if that function could only be restored. It might be argued that, if the animal is able to synthesise tryptophan in proteins, finding itself short for the "essential" function, it would synthesise protein, and use the tryptophan so produced to make up the deficiency. The argument falls on two counts: quantitative and teleological.

Tryptophan comprises some 2 per cent of the proteins containing it. So that if an animal is to keep up its tryptophan supply by using protein synthesised in its body, it must make fifty times that weight of tryptophan, as protein. A rat requires 20 mgm. of tryptophan per diem. To supply this, it would have to make 1 gram of protein, or say 10 grams of tissue, and then re-autolyse it all. And even then, the tryptophan seems to be of no use to the animal unless it passes through the liver before reaching the system, so that tryptophan from autolysis is not available.

And on the teleological score, the necessity for such machinery would never arise in nature. There is a considerable uniformity in the amino acid constitution of the proteins (see diagrams), and throughout the evolutionary development of animals dependent upon ingested amino acids for specific purposes, there would never be any danger of a lack in one essential amino acid alone. In nature this seldom occurs. In experimental feeding work it is difficult to achieve. It would never become necessary, therefore, while the specific function of an amino-acid

was developing, for the animal to evolve the machinery for making it on a large scale in the free state, or for transferring it from its normal site of synthesis in the proteins to the new site of utilisation. The position of the essential amino acids is, in this sense, exactly that of the vitamins, but on account of their more general distribution and the relative difficulty of excluding them from the diet, they have not received the same attention.

Schichiri and Sakata (1933) tried the effect of injecting tryptophan into animals on a tryptophan-deficient diet. They claim that there is a replacement, and the injected material is efficacious in relieving the symptoms. Their findings are supported by those of du Vigneaud, Sealock and van Etten (1931), but are in opposition to those of Jackson (1929). The same experiment has been repeated here (Alcock, 1934b) and the result is definitely negative. The injection of tryptophan in no way improved the condition of the animals on the deficient diet, and it would therefore seem that the function of tryptophan which makes it essential for life is linked up with the liver; that passage through the liver is essential before the function can be fulfilled. This evidence tells strongly against the view that the growth failure in tryptophan deficiency is to be ascribed to the lack of tryptophan available for protein synthesis. In these experiments the tissues were amply supplied with the amino acid, yet the animals were declining for the lack of it.

The conception of the dual function of tryptophan is thus clear, and equally clear is the absolute independence of the two tryptophans used for these two functions; there is no interchange. The study of the essential amino-acids, as exemplified by tryptophan, would thus argue that protein synthesis is independent of the make-up of the dietary amino acid complex, and supports, therefore, the view that the amino-acids in the proteins are actually made there.

What has thus been argued for tryptophan applies to the other essential amino-acids. In particular, lysine, which, although necessary for growth and development, is not essential for maintenance. During the period of lysine deficiency, the animal makes it insofar as it is a protein constituent, although it is unable to synthesise it for some purpose which renders it indispensable. Presumably the indispensability of histidine is to be associated with its purine function; throughout the period of histidine deficiency the animal makes globin and other histidine-containing proteins. It seems clear that both the essential and the non-essential amino-acids can be, and probably are, made in the tissues during the synthesis of the proteins there.

It would thus appear that what little evidence there is on the subject of protein synthesis in the animal body all points to the conception of that process which was outlined in the section on protein origins: that the amino acids in the blood stream are taken into the tissues where synthesis is in progress, broken down to some simple common group, analogous to that first synthesised by the plant, and that this material yields, first by simple polymerisation, a "skeleton" protein molecule, and secondly, by an internal rearrangement, a fundamental common parenchyma protein. The nature of the tissue asserts itself at this stage, and the specific protein, characteristic of the tissue, is then produced.

SUMMARY

The idea that the proteins are synthesised by an enzymic condensation of the amino acids is, it is claimed, untenable. Considerations of the possible evolutionary processes leading to the elaboration of proteins suggest that the synthesis of a primitive type of protein, from relatively simple materials, is a common attribute to living creatures. The possible composition of this protein is argued from the average amino acid make-up of a wide selection of known proteins.

A review of the scanty evidence from experimental work on protein synthesis in the growing tissue reveals no information, either supporting or opposing, this far-reaching protein synthetic process. The proteins would seem to be built from the blood amino-acids, but the experiments which establish this give no suggestion as to the course of the synthesis.

The supposed synthesis of proteins *in vitro* rely on the results of three observations. Taylor's synthesis of a protamine from amino acids is unequivocal, but it is not possible to argue from protamine synthesis to protein. Abderhalden's synthesis of tissue proteins from autolysed tissue is more open to doubt, as the nature of the product is not established, and the work has neither been put on a quantitative footing, nor confirmed.

Plastein formation, by pepsin and trypsin, indicates a condensation function on the part of these enzymes. The complexity of plastein has never been proved, and the supposed synthesis may be no synthesis in fact. If it is indeed a synthesis, it is unlikely that it has any parallel in the cell.

The only positive evidence comes from the work on the essential amino-acids. It is shown that an animal can, in all probability, synthesise proteins, even though it is receiving a deficient amino acid mix-

ture. In the case of the dispensable amino-acids, it might possibly compensate this by synthesising the missing member elsewhere, though this is unlikely. In the case of the essential amino-acids this is not possible, and the synthesis must necessarily go on in the tissues.

The evidence would suggest that the process of synthesis is as follows: the blood amino acids are taken up by the tissues, and broken down to some simple unit. This unit, by polymerisation, provides a basis on which the protein is built. Within this basis molecule a process of differentiation sets in and a definite proportion of each amino acid is formed, the protein so produced being similar in composition to the probable ancestral protein. The subsequent differentiation of the molecule, involving the addition or elimination of amino-acids, or the addition of prosthetic groups, follows a course determined by the nature of the tissue in which it is being produced.

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THE KINETICS OF HEMOLYSIS

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Whatever may have been the case in the past, the kinetics of hemolytic reactions are now of interest for three reasons. 1. Hemolysis is an extreme, and for that reason possibly the simplest case of altered permeability, and by studying it in detail we can obtain information, if not about how permeability is maintained, at least about how it is modified and destroyed. 2. Some lytic reactions are among the simplest cases of reactions between drug-like substances and the cell, and by investigating them we can learn something about the factors which may be expected to operate in more complex cases. 3. In the hemolytic systems we have an instance in which a quantitative study of a biological phenomenon yields results which are almost as reproducible as those met with in physical chemistry, and which can comparatively readily be described in the same sort of way as the physical chemist describes his results, i.e., by equations of various kinds. The questions of whether the equations have a real meaning or are partly empirical, and of whether biological phenomena can be expected to be described by other than empirical equations, need not be discussed here; it is sufficient to say that no biological phenomenon can be said to have been thoroughly explored until the observations are describable in equational form, empirical or otherwise, and that phenomena require to be thoroughly explored before they can be explained or understood.

It will be as well first to outline what is known about the cell upon which the hemolysins act. The mammalian erythrocyte is a balloon-like structure, consisting of a cell membrane or envelope enclosing hemoglobin, salts, and many other substances in solution. The membrane contains about 80 per cent of stromatin, a protein with an isoelectric point of pH 5.5, and about 20 per cent of lipoids, mainly lecithin and cholesterol (Jorpes, 1932). The idea has been advanced that the lipoids are arranged in a bimolecular layer, (Gorter and Grendel, 1925), but without sufficient evidence. The lipoids and protein are more probably disposed so as to form a membrane of a complex mosaic type. This membrane is normally permeable to water and to anions,

but not to cations such as Na and K, at least when the cell is bathed in normal plasma. Like all cell membranes characterized by semi-permeability, it is almost completely non-conducting and presents a static capacity of about 1 μF per cm.^{2}, the frequency dependence of which, curiously enough, is only about 2 per cent instead of about 20 per cent as in the case of the membranes surrounding most other kinds of cell (Fricke and Morse, 1925–26; Fricke, 1933). Fricke (1923–4a, b; Fricke and Morse, 1925–26) has calculated that the layer which exhibits the semi-permeability is about 0.003 μ thick, i.e., of the order of thickness of a film made up of a few molecules only. This layer, of course, forms only a part of the cell "envelope," which is much thicker and can be made visible by using dyes. There is indirect evidence that the membrane as a whole has something of a liquid crystal structure (Ponder, 1934a), and that this accounts for the biconcave shape of the cell. The interior is about as viscous as is olive oil at the same temperature, and there may or may not be a fine "stroma" or supporting sponge work, but it is more likely that there is a fluid cytoplasm, capable of gelation under certain circumstances. At all events, the idea that the mammalian red cell is a bag filled with salts, pigment, and water, inert, and dead or dying, is quite wrong, for it has a measurable metabolism of a rather complicated kind which is smaller than but otherwise similar to that of more fully organized cells (Ramsey and Warren, 1930, 1932, 1934).

I. Hypotonic hemolysis. The classical explanation for the hemolysis which occurs in hypotonic solutions is that the red cell, the interior of which has an osmotic pressure higher than that of the hypotonic medium, takes in water so as to establish osmotic equilibrium. This results in swelling, and if the swelling is sufficient the cell membrane is stretched to such an extent that hemoglobin escapes, and lysis results. In its qualitative form this explanation is now universally accepted, but it is usually amplified by adding that osmotic equilibrium is brought about by the *transferance of water alone*, the cell behaving as a "perfect osmometer," impermeable to the osmotically active substances which it contains. Suppose in the meantime that this is so, and let us set down the factors upon which the swelling at equilibrium in a hypotonic solution must depend. To simplify matters, suppose the hypotonic medium to be hypotonic plasma, and define tonicity as the ratio of the Δ of the hypotonic plasma to the Δ of the undiluted plasma. Then the tonicity of the undiluted plasma is 1.0, that of the plasma diluted with

an equal volume of water 0.5, and so on. The amount of swelling attained at equilibrium in the hypotonic medium will then depend on:

1. The osmotic pressure in the cell interior. This is usually taken as the same as that of the plasma normally bathing the cells (Collins and Scott, 1932). At one time errors in method gave rise to the view that there is a difference.

2. The quantity of "free" or "osmotically active" water in the cells. The total quantity of water is about 60 to 65 per cent by volume, but it was once thought that some of this is "bound" water. We now know that at least 94 per cent of it is "free" (Hill, 1930).

3. The tonicity of the surrounding medium. Equilibrium is reached between this and the cell interior when

$$V = 100 - RW + RW/T \tag{1}$$

where T is the tonicity of the medium, defined as above, V the volume of the cell at equilibrium, the initial volume in isotonic plasma being denoted by 100, W the quantity of water initially present in the cells and expressed as a percentage by volume, and where R is the fraction of the total water which appears "free" or "osmotically active," e.g., if all the water is "free," $R = 1.0$, if half of it is "bound," $R = 0.5$, and so on. This equation is correct only if the volume of the medium surrounding the cell is exceedingly great. If the volume is limited, the equation is more complicated, principally because of the units used; the equilibrium condition, however, is always of the form

$$\frac{RW}{RW + (V - 100)} = \frac{a\,\bar{T}}{a - (V - 100)} \tag{2}$$

a being a factor which is determined by the relative volume of surrounding medium.

As the tonicity of the surrounding medium is made less, the swelling of the cells increases in accordance with this equation, and so equilibrium is always reached with a larger cell volume than the initial one. If the hypotonicity is too extreme, however, the cell may become permeable to hemoglobin before equilibrium is reached, and so there is a maximum volume which is compatible with the integrity of the cell. This is called the "critical volume," denoted by V_h. Other things being equal, the greater the critical volume, the smaller is the tonicity of the external medium in which the cell can remain intact.

It will be apparent that both the form of the equilibrium equation

and the idea of a critical volume make it necessary that we shall be able to obtain actual measurements of red cell volume. There are no less than seven fairly reliable methods for measuring red cell volume, four of which give the volume in μ^3 while the remainder measure volume increases only.

If the degree of hypotonicity is not too great, the most reliable method is a modification of Stewart's *colorimetric method* (Stewart, 1899b; Ponder and Saslow, 1930a). It consists in mixing a known volume of a solution of hemoglobin in plasma with a known volume of whole blood, determining the extent to which the hemoglobin is diluted by the plasma in the whole blood, and calculating therefrom the amount of plasma present and thence the volume occupied by the cells. Division of the latter figure by the number of cells present gives the mean volume of the individual cell. If the volume in hypotonic solutions is required, hypotonic plasma and hemoglobin solution are added to the whole blood instead of hemoglobin solution alone (Ponder and Saslow, 1930b). Unfortunately the method depends on the cells not losing hemoglobin, and so can be used for measuring cell volume in normal or in slightly hypotonic plasma only.

The *diffractometric method* is based on the fact that mammalian red cells in saline, whether hypotonic, isotonic, or hypertonic, assume the form of perfect spheres without change in volume if placed between a slide and a closely applied coverglass (Ponder 1928–29), and that the mean radius, and therefore volume, of such spheres can be measured by determining the radius of the coloured diffraction patterns which arise when a parallel beam of light is directed normally to the slide (Pijper, 1919; Millar, 1926; Allen and Ponder, 1928; Ponder, 1933a). The method can be extended to cells in hypotonic and isotonic plasma because of the fact that red cells can be converted into perfect spheres, again without change in volume, by the addition of lecithin to the plasma surrounding them (Ponder, 1933a).

The most common method of measuring red cell volume is the *hematocrite method.* It is unsatisfactory for many purposes, however, because the height of the column of packed cells, and therefore the apparent volume of the individual erythrocyte, depends largely on the rate and duration of spinning (Millar, 1925a; Ponder and Saslow, 1930a). As a result, hematocrite determinations cannot be trusted to give more than approximate results for red cell volume in absolute units, although they are convenient for finding relative values.

Red cell volume can also be measured by *conductivity methods*, origi-

nally introduced by Stewart (1899a) and later elaborated by Fricke (1923a, b). So far these methods have not been used as much as they ought to be, principally because the biconcave shape of the cell introduces dubiety into the calculations. If the cells are converted into spheres by the addition of lecithin, however, the difficulty disappears, and the volume occupied by the cells can be easily determined. Division by the number of cells present gives the mean individual cell volume.

The three remaining methods (Macleod and Ponder, 1933) give values for the increase in red cell volume which occurs in any hypotonic solution, but do not give the volume in absolute units. The first depends on the fact that when a red cell increases in volume by taking in water, its density diminishes. The second depends on the fact that when the cell takes in water, the concentration of hemoglobin in it diminishes. The third is the most direct, and depends on the estimation of the quantity of water present in the cell before and after it has swollen, the quantity of contained water being determined by weighing a known mass of cells before and after drying. From the figures for density, hemoglobin concentration or water content, the percentage increase in volume of red cells suspended in a hypotonic medium can be calculated. (One calls the volume of the cell before it has swollen 100, and expresses the swollen volume as a percentage, e.g., 120. The difference between the two figures is the percentage increase in volume.) There are a number of other methods for measuring red cell volume, e.g., refractometric methods (Reich, 1921; Emmons, 1927–28), viscosimetric methods (Suzuki, 1921) and methods involving various analytical procedures (Bleibtren and Bleibtren, 1891–92; Hoppe-Seyler, 1893; V. Bunge, 1898; Emmons, 1927–28), but all of these are unreliable for one reason or another (Brooks, 1925).

If we were to make measurements of volume by any one of the above methods which may be applicable to the system under consideration, and if the cell were a "perfect osmometer" ($R = 1.0$, and equilibrium reached by water exchange alone), we should expect to find from expression (1) the following relation between the volume of the cell and the tonicity of the environment for the usual case in which the quantity of water in the cell is 60 per cent and where the volume of the cells is small compared with that of the surrounding fluid:

T	1.0	0.9	0.8	0.7	0.6	0.5
V	100	107	115	124	140	160

As a matter of fact, if the environment is hypotonic oxalated plasma or hypotonic NaCl, we usually find the following sort of correspondence instead:

T	1.0	0.9	0.8	0.7	0.6	0.5
V	100	103	108	112	120	130

The swelling is much less than it ought to be if the cell were a "perfect osmometer," and the problem is to explain the difference. Excluding, as we can safely do, the existence of any appreciable "back pressure" exerted by the stretched membrane, there are at least two explanations to be considered.

1. The abnormally small swelling might be due to the cell's containing an appreciable amount of "bound water," i.e., to R being less than 1.0. This has been suggested (Gough, 1924; see Ege, 1922a, b), but it can scarcely be accepted in view of Hill's (1930) demonstration that the "free water" is at least 94 per cent of the total. Moreover, before putting forward any such idea, it is well to bear in mind that a similar abnormally small swelling has been observed in muscle immersed in hypotonic solutions (Hill, 1930) and also in kidney tissue (Siebeck, 1912) and that the explanation that this is due to the presence of "bound water" in appreciable quantities has failed equally in these cases.

2. The second explanation is that the cell is not a perfect osmometer, impermeable to osmotically active substances, but that it reaches equilibrium with its environment by taking in water and also losing osmotically active substances (mainly cations) (Ponder and Saslow, 1931). More specifically, if the cell loses a quantity x of osmotically active substance, the equilibrium condition corresponding to (2) is

$$\frac{W - x}{W + (V - 100)} = \frac{a\,T}{a - (V - 100)}$$

and we have the interesting relation

$$dX/dT = \text{const}/R, \tag{4}$$

where X is x expressed as a percentage of the quantity of osmotically active substance originally present in the cells. For each downward step in tonicity, the cell accordingly loses a constant percentage of its contained osmotically active substance, and no more; if R is small, the percentage loss per unit step is great, and vice versa. At the same time

it takes in water, and swells in consequence. At the end of the time occupied by this rapid salt and water exchange, the cell is again in equilibrium with the fluid surrounding it; the equilibrium, however, has been reached by an intake of water and a simultaneous leakage of part of the contained salts, i.e., partly by a temporary loss of semipermeability to cations.

At first sight this may appear an extraordinary hypothesis, but the relation expressed in (4) arises, for reasons of mathematical necessity, from two facts: *a*, that the red cell increases in volume in many hypotonic solutions as if about half or even less of its contained water were "free," and *b*, that nearly all of its water is in fact "free." Let us look at the idea in the light of other evidence. In hypotonic solutions of glucose, in which the swelling of the cells is conspicuously small, there is general agreement that cations are lost from the cells (Bang, 1909; Joel, 1915; Kerr, 1926a, b, 1919; Ponder and Saslow, 1931; Jacobs, 1932; Ponder and Robinson, 1934a; Davson, 1934), and they can be detected in the surrounding medium by a variety of methods (conductivity measurements, direct chemical analysis, etc.). In the case of red cells in hypotonic NaCl and KCl, Kerr, Davson, Ponder and Robinson, and others have shown by analytical methods that cations escape, and in the analogous case of muscle, the swelling of which is also abnormally small in hypotonic solutions, leakage of cations into the surrounding medium has been not only detected but even estimated with good quantitative agreement. The same thing is true for kidney tissue (Siebeck, 1912), which also exhibits an abnormally small swelling in hypotonic solutions, and the abnormally small swelling of the injured Arbacia egg (McCutcheon and Lucké, 1932) can be accounted for in the same kind of way.

Under certain circumstances, however, the red cell appears to behave as a perfect, or nearly perfect, osmometer, and the condition which seems to be necessary for this is that the medium in which it is suspended shall not differ substantially from the plasma which normally surrounds it (Ponder and Robinson, 1934b). Red cells from defibrinated blood, for instance, behave as very nearly perfect osmometers ($R = 0.8 - 0.9$) when placed in slightly hypotonic serum derived from defibrinated blood. But if the tonicity of this serum is made less than about 0.75, the swelling is less than one would expect from simple osmotic laws, and the cell becomes an imperfect osmometer which loses salts into the medium. Dilution of the serum beyond a certain quite small amount thus introduces what can be called a "leakage

factor." If oxalate is used as an anticoagulant, a leakage factor is introduced at the very beginning, and the cell behaves as an imperfect osmometer in oxalated plasma of any degree of hypotonicity. Similarly, the replacement of the normal plasma surrounding the cell by hypotonic solutions of NaCl, KCl, and glucose, etc., introduces a leakage factor, and in such solutions the cell is an imperfect osmometer. The state of affairs, indeed, is very much like that described for the Arbacia egg, which is a perfect osmometer only as long as it is uninjured, and, if it makes the idea any easier of acceptance, it can be conceded that such treatment as introduces leakage factors in the case of the red cell inflicts "injuries" upon it.

If the conditions which govern the amount of water which enters the cell at equilibrium are complicated, those which are concerned with the rate of entry of water are even more so. As equilibrium is reached within a matter of seconds, one cannot measure the increase of cell volume in a hypotonic solution from instant to instant, at least by any method yet known, and so one has to make the assumption that some certain degree of hemolysis, e.g., just commencing hemolysis or 50 per cent hemolysis, corresponds to the cells having swollen to a certain definite volume. There is reason to think that under the majority of conditions this assumption is a correct one. One then measures the time taken for such a degree of hemolysis, i.e., for this volume to be reached under various conditions, this being a comparatively simple matter, as there are many ways of measuring percentage hemolysis.

The fundamental equation for the study of rates is (Jacobs, 1932)

$$dV/dt = kA(c - C) \tag{5}$$

which states that the rate of entry of water, or of increase in volume, is proportional to the cell area A and to the difference between the concentration of osmotically active substances $(c - C)$ inside and outside the cell respectively. If the volume of the cells is very small compared with the volume of the surrounding medium, and if we suppose that because of its peculiar shape the red cell can swell appreciably without its surface area A increasing, we can obtain from (5)

$$k't_h = \frac{c_o}{C^2} \log \frac{c_o - C}{c_o - rC} - \frac{r - 1}{C} \tag{6}$$

in which c_o is the initial osmotic concentration of the cell interior, t_h the time for some selected degree of hemolysis to be reached, r the

ratio c_o/c_h, where c_h is the concentration in which the selected degree of lysis would just be reached in infinite time (i.e., at equilibrium), and k' a constant, equal to kA/V. If $C = O$, and the cells are placed in distilled water, (6) becomes

$$k't_h = \frac{1}{2c_o} \cdot (r^2 - 1) \tag{7}$$

and only if the rate of entry water is described by these equations can we say that the cell is obeying "simple osmotic laws."

The first point which calls for remark is that it is possible for equations (6) and (7) to be followed at the same time as equations (3) and (4), for the method of measuring the rate of intake of water is necessarily an indirect one, consisting in showing experimentally that k' is constant under a number of different conditions, or nearly so. Since $k' = kA/V$, where V is the volume occupied by the "free" water of the cell, it is immaterial so far as the constancy of k' is concerned whether there is "bound" water or not, or leakage or not, or whether $R = 1.0$ or any other figure. The equations for rate of water intake are accordingly not affected by the rapid leakage of salts, which, in order to avoid the fiction of "bound" water, we have to imagine as occurring simultaneously with the intake of water, and when one uses the terms "perfect" or "imperfect" osmometer, one has to bear in mind that an osmometer which is "simple" as regards *rate* of water intake may be far from simple when the *volume* of water taken in is considered.

Our knowledge regarding the extent to which these simple equations for rate of water intake describe the actual behaviour of the red cell is almost entirely due to Jacobs and Parpart (Jacobs, 1930; Jacobs and Parpart, 1931; Jacobs, 1932; Jacobs and Parpart, 1932a, b) and may be summarized by saying that there are so many factors which modify the osmotic behaviour of the cell that the equations are applicable only in a general sort of way.

1. In very hypotonic media in which lysis occurs within a few seconds, the equations describe the results satisfactorily except if the salt concentration is exceedingly small, when irregular results are obtained.

2. In solutions of non-electrolytes, in which lysis takes place in longer than 2 to 3 seconds, but in less than 30 seconds (i.e., in which the lysis is neither very rapid nor quite slow), there is a sudden increase in red cell resistance over that observed when lysis takes a very short time. Jacobs and Parpart account for this by pointing out that, in non-electrolyte media, there may be an exchange of anions from the cells

for OH ions from the medium, the resulting increase in base-binding power of hemoglobin causing a decreased osmotic pressure within the cells. The difficulty is that the argument depends on the cation impermeability and other properties which the cell exhibits in plasma remaining unchanged when it is placed in very hypotonic solutions. At all events, it is clear that in non-electrolyte solutions there is considerable departure from simple osmotic behaviour.

3. Both changes in temperature and changes in pH appear to affect the rate of water intake, and also the final equilibrium which is reached. Jacobs and Parpart regard these effects too as being due to changes in the base-binding power of hemoglobin, and so derive the equation in which r is the ratio of base in the cell to the cell hemoglobin, F_1

$$\frac{W_1}{W_2} = \frac{2r + 1 - F_1}{2r + 1 - F_2} \tag{8}$$

and F_2 the amounts of base bound by unit quantity of hemoglobin under two conditions corresponding to the presence of water W_1 and W_2 in the cell (e.g., two different pHs), and where the concentration of salt in the suspension medium is constant. This equation allows them to predict experimental results with a reasonable degree of accuracy. Again the difficulty lies in the treatment assuming the cells to have a complete cation impermeability at different temperatures, different pHs, etc., when the complete cation impermeability does not exist under the conditions of the experiments; it is possible, however, that the cation impermeability, although incomplete in the sense of equation (4), may be sufficiently constant to give the method of treatment validity.

4. In experiments lasting some hours, Jacobs and Parpart have shown that a leakage of salts occurs in the case of some kinds of red cell (rabbit and man), but not in the case of others (e.g., ox). This leakage, however, is something over and above that postulated in equations (3) and (4), which occurs about as readily in the case of ox cells as in the case of any other kind; the reader must be careful not to confuse slow loss of salts, which have been the subject of many experimental investigations in the past (e.g., Joel, 1915; Hamburger, 1891, and some of Kerr's experiments (1929)) with the rapid leakage which has to be postulated in order to account for the volume changes.

Let us now consider the two classical problems connected with hypotonic hemolysis. 1. Why is it that hemolysis occurs in different concentrations of different electrolytes and non-electrolytes, even if these

concentrations are prepared so that the osmotic pressure of the different solutions is the same? This fact has always given rise to difficulty; sometimes it has been left unexplained, sometimes it has been accounted for on the now abandoned hypothesis of "bound water" (which would have to vary in amount with every different substance in which the cells were suspended), and sometimes the whole osmotic theory has been abandoned because of it. 2. Why is it that, in hypotonic solutions of the same electrolyte or non-electrolyte, the cells of one animal are more "fragile" or less "resistant" than those of another? This fact has never been adequately explained.

1. When red cells are placed in a hypotonic medium they swell until they attain a certain critical volume, V_h, and then they hemolyse. The amount to which they swell in a solution of any given tonicity depends on the water content of the cell, the initial osmotic pressure of its interior, and on the extent to which the cell behaves as a perfect osmometer. When we are considering the swelling of the cells of the same sample of blood in hypotonic solutions of different substances (e.g., NaCl, KCl, glucose, etc.), the water content of the cells is constant, and we can start off with solutions of the different substances which are osmotically equal to the plasma normally surrounding the cells and therefore to the red cell interior. The tonicity of these solutions can be put equal to unity, and the hypotonic solutions of the different substances regarded as having a tonicity of 0.9, 0.8, etc., a tonicity of 0.5 (say) for any one substance having the same osmotic pressure as one of tonicity 0.5 of any other. The swelling in any given hypotonic solution then depends only on the perfection of the osmometer, and this we express by the value of R, saying, in effect, "Our cell, which we recognize as an imperfect osmometer with virtually all its water 'free' (i.e., $R = 1.0$), behaves as a perfect osmometer containing only half (say) of its water in a 'free' state (i.e., $R = 0.5$)." The greater R, the more will the cell swell in a solution of given hypotonicity. But it can swell and remain intact only until it attains its critical volume; there must accordingly be some tonicity corresponding to V_h, and this tonicity, T_h, will be the lowest one in which the cell can reach equilibrium and remain intact. If the cell is a perfect osmometer, T_h will be larger than if it is an imperfect osmometer; in fact, other things being equal, T_h will depend on the value of R.

The key to the problem of why different values of T_h are found for different substances prepared so as to be osmotically equivalent lies in Jacobs' idea of critical volume (Jacobs, 1930), for it can be shown

that the critical volume is substantially the same whether the cell is surrounded by hypotonic NaCl, KCl, LiCl, glucose or a number of related substances (Ponder and Saslow, 1931). The usual value, for the rabbit red cell, is about 140, the normal volume of the cell being denoted by 100. So if V_h is constant, the different values of T_h observed with the different substances (e.g., 0.52 for NaCl, 0.60 for KCl, 0.44 for glucose, etc.), must be due essentially to a variation in the value of R for the different substances, the cell behaving as a more perfect osmometer in NaCl, say, than in glucose.

This hypothesis, involving the relation between the three variables T_h, V_h and R, has been tested and found to be fairly satisfactory, for given V_h (measured diffractometrically) and R (obtained from a value for the swelling in a solution of tonicity greater than T_h), the value of T_h in solutions of a whole series of different substances can be predicted with quite considerable accuracy. The difference in the fragility of red cells suspended in osmotically equivalent hypotonic solutions of various substances accordingly has nothing to do with "fragility" or "resistance" in the ordinary sense, but depends mainly on the fact that leakage of osmotically active substances is greater in solutions of some substances than in solutions of others (Ponder and Saslow, 1931; Ponder and Robinson, 1934). In the case of non-electrolytes, the explanation of the different fragilities may be further complicated by changes in the base-binding power of hemoglobin, and therefore of the osmotic pressure in the red cell interior, occurring.

2. The reason why the cells of one animal hemolyse at a different tonicity of a particular substance than those of another animal, on the other hand, is that there are differences in the critical volume which different kind of red cells can attain (Ponder, 1934b). The red cells of man, the rabbit, the ox, and the sheep, for instance, are all about equally imperfect osmometers in hypotonic NaCl or hypotonic serum, but they exhibit different fragilities, and the value of T_h is very different for the four kinds of cell. This is because the critical volumes are different. Calling the normal cell volume 100 in each case, the critical volume for the cells of the sheep is about 126, that for the cells of the ox about 130, that for rabbit cells about 137, and that for the cells of man nearly 146. The greater the critical volume, the greater is the degree of hypotonicity with which the cell can come into equilibrium; other things being equal, T_h is accordingly less for the cells of man than for those of the rabbit, less for the cells of the rabbit than for those of the ox, and so on.

Why the red cells of one animal should be able to reach a greater critical volume than those of another is difficult to explain. Brinkman and van Dam (1920) have put forward an explanation in which the different fragilities are accounted for in terms of differences in the cholesterol/lecithin ratio in the cell membrane, but their experimental evidence has not been confirmed (Saslow, 1932). Some indication, however, is given by the relation which appears to hold between initial red cell volume V and the extent to which the area A can be stretched without the cell hemolysing, for if one plots these two variables against one another one gets a very good straight line. Thus it seems that dA/V = constant, which is a new way of putting an old observation, that the "bigger" the cell, the greater is its resistance (Krumbhaar, 1928). The linear relation, however, is one between stretching and initial volume, and not one between stretching and initial area. If we think of the red cell membrane as a fluid or semi-fluid film, the semi-permeability of which depends on a thin layer only a few molecules thick, the existence of such a relation suggests that when the membrane as a whole is stretched, its integrity is maintained by molecules, originally not in the layer, entering it so as to fill up the gaps produced by the stretching. The stretched semi-permeable layer is thus able to "repair" itself, up to a point, by drawing on "reserves" from the cell interior, or, more likely, from other layers of the cell membrane. This idea, of course, is somewhat speculative, but there is at least one piece of evidence in favour of it; the capacity of the membrane does not change as the cell area increases in hypotonic solutions, which means that while the membrane is stretched, it is not thinned (Fricke and Curtis, unpublished).

To summarize, the fragility of the cell is affected by at least four factors; its water content, the osmotic pressure of its interior, the critical volume which it can attain, and the extent to which it behaves as a perfect osmometer. The first two factors are usually of secondary importance. The third factor, the critical volume, depends principally on the type of cell, and the fourth factor principally on the nature of the hypotonic environment. When fragility is measured in the usual way, by putting red cells into hypotonic NaCl and observing the concentration (which does not give the true tonicity, unless the Δ of the plasma normally surrounding the cells is known) in which they hemolyse, the results may mean anything, and more usually nothing, for such measurements of fragility amount to measurements of as many as four variables at one and the same time.

II. Simple hemolytic systems. 1. *The "normal" kinetics.* For purely practical reasons, it is usual to distinguish between simple and complex hemolytic systems, a system being called simple when the lysin is a single substance, e.g., saponin, which acts directly on the red cells, and complex when lysis is produced, not by a single substance, but by two or more substances acting together, e.g., complement and amboceptor. The kinetics of hemolysis in the simple systems are usually the least complicated, and as an example we can take the case of saponin acting on washed human red cells at a constant temperature, e.g., 25°C.

In such a case it is easy to satisfy one's self that the reaction which results in lysis is accompanied by a using-up of lysin (Ponder, 1934c), presumably by reason of a combination between the lysin and some component of the red cell membrane, possibly the protein "stromatin" of Jorpes (1932). Denote by x the quantity of saponin so used up, and the initial quantity of lysin by c: supposing the quantity of the membrane component to be relatively great, we then have for the velocity of an irreversible reaction between cell and lysin (the "fundamental reaction"),

$$dx/dt = k(c - x)^n. \tag{9}$$

Here k is an "apparent velocity constant," and n is a constant which regulates the order of the reaction. At one time it was thought that $n = 1.0$ always (Ponder, 1927), but recent investigation has shown that it is usually greater than unity, common values being such as 1.7 or 2.2 (Ponder and Yeager, 1930a). It has been suggested in explanation that the lysin exists in aggregates of various sizes, when n would have the meaning

$$\frac{\text{Mean no. of lysin molecules combining with one cell molecule}}{\text{Mean no. of molecules per aggregate}},$$

and its value would not usually be a simple integer. Whether this explanation is right or not is not of first importance in the meantime, and it now appears more likely that the necessity for introducing this constant arises because equation (9) is an expression of a stoichiometric reaction, whereas the reaction under consideration is of the class known as "pseudo-adsorption."

By integration of (9) we get

$$kt = \frac{p}{p-1}\left\{c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}}\right\} \qquad (10)$$

in which t is the time in minutes for completion of hemolysis (or for reaching any given degree of lysis), c the initial quantity of lysin present, x the value of c when $t = \infty$ or, in practice when $t = 300$ minutes or thereabouts, and where $p = 1/n$. This equation supplies a relation between any concentration of lysin, or dilution of lysin $\delta = 1/c$, and the time which it takes to produce complete hemolysis, on the supposition that complete lysis results from transformation of a certain constant quantity x of lysin, i.e., it is the equation of the *time-dilution curve*, obtained by plotting these variables against one another. Whether the reasoning by means of which the equation is derived is correct or not, it is remarkable how well the values of t calculated from it agree with the values observed experimentally.

If c is regarded as constant in expression (10), we can also obtain the quantity of lysin combined with the cells at the end of any time t, but the equation as it stands does not give us the amount of hemolysis which results from such a combination. The reason for this is that the cells offer different resistance to the lysin, the least resistant cells hemolysing when only a small quantity of their cell component is transformed as a result of union with the lysin, whereas the more resistant cells require larger quantities of their component to be transformed before they hemolyse. As we should expect the cells, when grouped according to their resistances, to be grouped according to some type of frequency curve, let us call the distribution

$$N = N_o \cdot F(x) \qquad (11)$$

in order to leave its form undefined in the meantime. As the fundamental reaction proceeds according to expression (10) quantities of lysin x_1, x_2, x_3, etc., combine with the cells in times t_1, t_2, t_3, etc. Those cells which have a resistance which corresponds to x_1 hemolyse when x_1 is formed (i.e., at t_1) and their number can be found from expression (11). When x_2 is formed the cells whose resistance corresponds to x_2 hemolyse, and again this number is given by expression (11); added to the number hemolysed at time t_1, it gives the total hemolysed at time t_2. And so on. To find the numbers hemolysed at the end of any time up to the time

for complete lysis, we have accordingly to solve the simultaneous equation,

$$kt = \frac{p}{p-1}\left\{c^{\frac{p-1}{p}} - (c-x)^{\frac{p-1}{p}}\right\} \qquad (12)$$
$$N = N_o \int_o^x F(x)dx$$

the limits of the integral being taken, as usual, from an origin at $x = 0$. In practice the solution is obtained graphically, and the curve relating the number of cells hemolysed to the time, measured from the beginning of the lytic reaction up to its completion, is always S-shaped and unsymmetrical. The S-shaped form is due to the second part of the simultaneous expression (12) being S-shaped, and the asymmetry is partly due to the fundamental reaction slowing down as the lysis goes on, and partly due to the frequency distribution of red cell resistances being itself asymmetrical.

Many methods have been devised for obtaining these S-shaped *percentage hemolysis curves*, (for N, the number of cells hemolysed, is most conveniently expressed as a percentage of the total number), and all, except a few comparatively useless methods which are based on direct counting, make use of the fact that as a cell suspension undergoes more and more hemolysis, it becomes more and more translucent. The amount of light transmitted by the suspension can be measured by simple opacimeters, in which an object, such as the filaments of a lamp, becomes visible through the suspension when the latter has reached a certain degree of translucency (Jacobs, 1930) by thermopiles, radiometers (Ponder, 1923), photoelectric cells (Ponder and Yeager, 1930a; Kesten and Zucker, 1928–29a) or special photometers such as the Stufenphotometer (Ponder, 1932a). All these methods show the percentage hemolysis curves to be S-shaped, and, when properly analyzed, to have the form demanded by the theory outlined above.

These views regarding the kinetics of lysis in simple hemolytic systems are comparatively recent. The study of the kinetics properly begins about thirty years ago with the papers of Arrhenius and his pupils (Arrhenius, 1915), and is linked up with a long series of studies of the allied phenomena of bacteriolysis and disinfection (Kronig and Paul, 1897; Madsen and Nyman, 1907; Eijkman, 1912; Chick, 1908, 1910; Paul and collaborators, 1909, 1910a, b, c). The characteristic of the Arrhenius school is the idea that the reaction between the cells

(erythrocytes or bacteria) and the lysin or disinfectant is a "monomolecular" one, described by the equation

$$dN/dt = k(N_o - N) \qquad (13)$$

in which N is the number of cells destroyed at time t, and N_o the number of cells initially present. The cells, in fact, are treated as if they were molecules of a reactant. If this expression were correct, percentage hemolysis curves would be uniformly concave to the t-axis instead of S-shaped. Such a result was actually obtained by obsolete methods, and appeared to support the "monomolecular" idea; regarded in the light of modern experiment and theory, however, the hypothesis is virtually untenable. Brooks' paper, published as early as 1919, contains arguments weighty enough to dispose of earlier investigations on the subject, and his theoretical conclusions have received ample experimental verification.

By methods identical with those used for investigating the kinetics of hemolytic systems containing saponin it can be shown that the kinetics in systems containing digitonin, the other hemolytic glucosides (senegin, sapotoxin, smilacin, helleborein, etc.) sodium taurocholate, sodium glycocholate, and the soaps, if at the right pH, are essentially similar, and are described by the equations of expression (12). The same expressions also describe the kinetics in systems containing bacterial hemolysins (Ponder and Maclachlan, 1927). Some lysins give anomalous time-dilution curves under special circumstances, and particularly if the pH is not between 6.0 and 7.0, but these cases will be discussed later; speaking broadly, however, all simple hemolysins yet investigated present the same "normal" kinetics.

2. *Inhibition and acceleration.* A large number of substances, when added to systems containing simple lysins such as saponin or the bile salts, bring about either inhibition or acceleration of hemolysis.

a. *Plasma and serum.* Since the reaction which results in hemolysis in the case of lysins of the saponin or bile salt class appears to be one between the lysin and a protein component of the red cell envelope, one would expect that these same lysins would react with other proteins to form non-lytic compounds, and that the addition of such proteins to hemolytic systems would result in the inhibition of hemolysis. The addition of serum or plasma has such an effect, and the effect is principally due to the contained proteins (Bayer, 1907; Ponder, 1924). It was once supposed that the inhibition is due to the contained lecithin

and cholesterol (Ransom, 1901; Stocks, 1919–20); it is true that they exert a slight inhibitory effect, but not nearly sufficient to account for the inhibitory effect obtained with serum or plasma.

The quantitative aspects of the inhibition have been extensively investigated (Ponder, 1924, 1925, 1932a; Ponder and Gordon, 1934), but are still insufficiently understood in that we have to resort to empirical formulae to describe them. We suppose that the lysin reacts with the added protein to form a non-lytic compound, and that the quantity of lysin rendered inactive by such a combination is rendered inert irreversibly. Then, if Δ is the quantity rendered inert, it seems that

$$\Delta = \alpha c_1^{1/\mu} \tag{14}$$

in which c_1 is the initial quantity of lysin present in the system, and where α and μ are constants, the latter always being > 1.0. At one time it was thought that this was an instance of "adsorption," since the expression somewhat resembles the adsorption isotherm, but this idea is now abandoned.

b. *Non-electrolytes.* Suspensions of red cells in isotonic sugar solutions are always more resistant to saponin hemolysis than are suspensions of the same cells in NaCl, etc., but the inhibitory effect varies with the sugar used and is not the same for the cells of all species, the order of effectiveness of the various sugars being different for the red cells of man, the rabbit, the sheep, etc. (Ponder and Yeager, 1928). In the case of any one kind of cell and any one sugar, however, the inhibition is described in a relatively simple way, for it appears that the resistance of the cells is increased in the sugar solution, but that the fundamental reaction is altered in no other essential way (Yeager, 1929). It can also be shown that the effect of the sugar, which becomes greater as the quantity of sugar is increased, is principally on the cells themselves, although the activity of the lysin is also slightly reduced (Ponder, 1926). It is known, of course, that the replacement of NaCl in a system by isotonic glucose causes changes in red cell permeability, cations being lost by the cells in detectable quantity (Bang, 1909; Joel, 1917; Kerr, 1929; Ponder and Saslow, 1931; Jacobs, 1932) and that replacement of the electrolyte by sugar may even result in partial hemolysis. This shows that the sugar has a direct effect on the cell membrane. While it tends to render the membrane permeable to hemoglobin, however, it also renders it resistant to saponin; in fact, the indications are that if all the electrolyte could be replaced by non-electrolyte, saponin

would be non-lytic, just as is eel serum when all electrolyte is removed (Gengou, 1909).

c. *Electrolytes.* If the effects of non-electrolytes are complex because they depend both on the kind of sugar and on the kind of cell, the effects of electrolytes such as NaCl, KCl, etc. (in isotonic solution) are far more complex in that they depend on the type of cell, the type of electrolyte, the concentration of electrolyte, and the concentration of lysin. The effects vary in a quite incomprehensible manner with the quantities of electrolyte introduced, and the order of the fundamental reaction may change as the amount of electrolyte is increased, sometimes increasing, sometimes decreasing, and sometimes passing through a maximum or minimum corresponding to some particular quantity of electrolyte. As the results are different for each monovalent kation, it is impossible to summarize them in a review of this kind, but I refer the reader to a paper by Gordon (1933a) whose conclusion is that no concept such as Hofmeister Series, molecular weight, etc., taken singly, can explain the experimental results.

d. *Effects of pH.* These are scarcely less complex. In the case of saponin, changes of pH between pH 5.1 and pH 7.1 have little effect. If the pH is less than 5.1, there is acceleration of hemolysis, and if it is greater than 7.1, there is inhibition, the amount of which varies, however, with the dilution of lysin employed (Bodansky, 1929; Gordon, 1933b). In the case of the bile salts, variations of pH are apt to result in the appearance of "hemolytic zones," i.e., in lysis being very much delayed in certain concentrations of the lysin. Under these circumstances, the time-dilution curve shows lumps upon it, instead of being uniformly concave to the t-axis, and expression (10) fails to describe its course in the region of the lumps. In the case of sodium taurocholate, the zones occur if the pH is greater than 8.1 or less than 6.0, but between these limits the time-dilution curves are of the usual regular form. In the case of sodium glycocholate, zones appear when the pH is greater than 6.0 or less than 4.1. Apart from the appearance of the zones, we have a set of very elaborate acceleration and inhibition phenomena which depend not only on the pH but on the dilution of lysin in the systems (Gordon, 1933b).

e. *Effect of temperature.* The effect of temperature on the velocity of hemolysis has so far defied all formulation, although, in general, lysis becomes more rapid as the temperature is raised. It is usual to express the effects of temperature in terms of the Arrhenius equation, and to find a "temperature coefficient," or a "temperature charac-

teristic" for the system under consideration, but in the case of hemolytic systems a peculiar difficulty arises, viz., as the temperature is increased, n, x and k in expression (6) all undergo change, and not the velocity constant alone (as is assumed in the use of the Arrhenius equation). Although the time-dilution curves and the variation in the constants are quite regular, the result is that plotting $1/T_{abs.}$ against log $1/t$ gives, not a series of straight lines with the same slope, but a series of lines with different slopes for each dilution of lysin, or even worse, a series of curves which show maxima and minima. In the case of saponin, for instance, the value of the "temperature coefficient" might be anything from 27,000 to 35,000, according to the dilution of lysin employed (Ponder and Yeager, 1930b); in the case of propyl alcohol, it might lie between 11,000 and 50,000 (Gordon, 1932), and in the case of sodium taurocholate, it might be either positive or negative (Ponder and Yeager, 1930b). In fact, the effect of temperature is too complex to be analyzed in this way.

3. *Resistance series.* Few contributions to the subject of hemolysis have attracted so much attention as that of Rywosch (1907), which contains the first record of the fact that the series obtained by writing down the names of common laboratory animals in order of their resistance to saponin is almost the reverse of that obtained by writing them down in order of their resistance to hypotonic saline. This can be seen at once if we place the two series side by side, the animal with the most resistant cells appearing first.

Saponin	*Hypotonic saline*
Sheep	Guinea pig
Goat	White rat
Ox	Dog
Cat	Grey rat
Grey mouse	Rabbit
Pig	Pig
Grey rat	Grey mouse
Dog	Cat
White rat	Ox
Rabbit	Goat
Guinea pig	Sheep

The inverse relation between the order of resistance to saponin and that to hypotonic saline is just what we would expect if there were some one factor determining the resistance, operating in one sense with respect to the one lysin and in the opposite sense with respect to the other. As early as 1910 Port pointed out that the order of resistance

to saponin is the same as the order obtained by writing down the animals according to the phosphoric acid content of their cells, and both Höber and Nast (1914) and Orahovats (1926) employ his idea. The resistance, however, becomes less as the quantity of phosphoric acid becomes greater, and this precludes the possibility of the lysin's reacting with the phosphoric acid or the substance from which it is derived. Yagi (1911), Fabre and Simmonet (1926), Brinkman and van Dam (1920) and others believe that the "single factor" which determines resistance is cholesterol or the lecithin/cholesterol ratio. The evidence for the "single factor" idea, however, is very slight indeed, for it can be shown that the fundamental premise itself is wrong, and that Rywosch's inverse series for lysis by saponin and hypotonic saline breaks down completely when the resistance of cells of animals other than those commonly used in laboratories is determined and tabulated (Ponder, Saslow and Yeager, 1930).

Further, there are almost as many resistance series as there are hemolysins, and every new lysin seems to give a different series, even when the lysins are closely related in nature (Ponder and Maclachlan, 1927; Kofler and Lázár, 1927; Yeager, 1928). As a result, any hypothesis which demands a "single factor" as determining red cell resistance is untenable. We can, of course, imagine that each lysin attacks a different component of the cell membrane, but one has the uneasy feeling that we have only to investigate the resistance series corresponding to a sufficiently great number of lysins in order to add to the supposed components of the membrane indefinitely. A far better way of getting out of the difficulty is to recognize that the effect of a hemolysin depends not only on the nature of the lysin, but also on the nature of the cell which it attacks, and to account for the multiplicity of resistance series in the following way. Let the lysin react with a component S in the cell membrane, so that, when lysis is complete, a quantity S_1 is transformed. A new substance, consisting of a combination of S and lysin, then takes the place of S as a component of the membrane; call this new substance S_2. The permeability of the membrane will then be a function of a, the quantity of S_2 formed, and b, the nature of S_2, which will clearly depend on the nature of the two substances from which it is formed, i.e., S and the lysin. The resistance of the cell will thus be a function both of the type of cell and of the kind of lysin, and this will result in there being a different resistance series for each hemolysin. The occurrence of such different resistance series, although rather extraordinary at first sight, is accordingly much what we would expect.

This, of course, is only an outline of the way in which different resistance series are accounted for, and for a fuller description the reader must consult the original papers.

4. *"Absorption" of lysins.* Lytic reactions cannot possibly proceed anywhere else but in the region of the red cell surfaces, and so it is the concentration of lysin which exists there, rather than in the system as a whole, which matters; our ideas regarding the kinetics of these reactions have accordingly to take account of the fact that most lysins are concentrated at red cell interfaces (Ponder, 1934c). A study of the "absorption" shows that it may be of two kinds: "initial" absorption, which takes place exceedingly rapidly, and "delayed" absorption, which continues throughout the course of the lytic reaction. The bile salts and the soaps show initial absorption, about one-third of the total lysin becoming concentrated at the cell surfaces within a few seconds. In this way what can be called an "external system" and an "internal system," concentrated near the cell surfaces, are formed, and it is within the latter that the lytic reactions go on. A great deal of experimental work will be needed before the relations between these "external" and "internal" systems are fully understood, and perhaps the most remarkable thing about them is that, once they are formed, they seem to behave almost independently of each other. In the case of certain other lysins, on the other hand, there is no initial absorption, but merely a slow disappearance from the fluid surrounding the cells as time goes on. Quantitative investigation of the course of this slow disappearance in the case of saponin has shown at least two interesting points, that the stage of complete hemolysis always corresponds to the disappearance of approximately the some quantity of saponin, and that the disappearance continues long after lysis is complete. The first point is in agreement with the preceding theory, and the second shows that the lysis of the cells is followed by a continuation of the lytic reaction in the form of stromatolysis, or fragmentation of the ghosts.

III. Complex hemolytic systems. By a complex hemolytic system is meant one in which lysis is produced only after the cells have been sensitized by a more or less specific sensitizing agent. Many such systems are known, but the kinetics of only a few of them have been investigated, and even these investigations are far from complete.

All the complex systems differ from the simple systems already discussed in one very striking way; the result obtained depends largely on the *order* in which the components of the system are added. For example, if we take 1 cc. of a cell suspension, add 0.1 cc. of serum, and,

a minute later 1 cc. of a 1 in 1000 taurocholate, the result is almost complete inhibition of hemolysis. But if we reverse the process, and add to the same quantity of cell suspension the same amount of taurocholate, and, after a minute, the same amount of serum, we get almost instantaneous lysis. The result thus depends on the order in which the components are mixed: we accordingly distinguish between a cell-serum-lysin system (for example) and a cell-lysin-serum system, the order in which the components are written down denoting the order of addition.

The few complex hemolytic systems which have been studied bear certain similarities to each other. The best known is that containing cells sensitized with a specific immune body or amboceptor, together with the substance known as complement. The kinetics of hemolysis in such systems is fairly well understood. The next type of system is one in which the specific sensitizing agent amboceptor is replaced by the non-specific sensitizing agent colloidal silicic acid, which, like immune body, sensitizes the cells to the lytic action of complement (Landsteiner and Jagic, 1904). The kinetics of lysis in these systems have been investigated, and they are considerably more complicated than those for lysis in cell-amboceptor-complement systems. Next we have those systems in which a non-specific sensitizing agent is supplied by brilliant green or one of the other tri-phenyl-methane dyes, and in which the lysin is serum or plasma protein instead of complement (Browning and Mackie, 1914; Mackie, 1919). In many respects, these systems are more difficult to investigate than are colloidal silicic acid-complement or amboceptor-complement systems, and our knowledge of their kinetics is little more than qualitative. Finally there is the system in which one of the bile salts sensitizes the cells to the action of plasma proteins, so that plasma, added after the bile salt has been in contact with the cells, produces rapid lysis. From the standpoint of kinetics, this is the most complicated of all the complex systems known, unless it be for the system containing snake venom and complement, the kinetics of which have not yet been investigated.

1. *Amboceptor-complement systems.* If cells are sensitized with a constant amount of amboceptor, the time for complete lysis increases in a regular manner as the concentration of complement is decreased, just as it increases with decrease in the concentration of a simple hemolysin. The result of plotting the time for complete lysis against the dilution of complement initially present in the system is a typical time-dilution curve, described by expression (10) with a value of n of about

unity. Percentage hemolysis curves can also be obtained by measuring the percentage of lysis corresponding to different times, and these can be fitted in the same way as can the percentage hemolysis curves for a simple lysin (Ponder, 1932b).

The particular time-dilution curve obtained depends, however, on the quantity of amboceptor used for sensitizing the cells, for, in general, the smaller the quantity of amboceptor used, the slower is the lysis which ensues on the subsequent addition of complement. Since the time-dilution curve corresponding to any one quantity of amboceptor can be analyzed completely in terms of three constants, n, x and k, we can investigate the way in which these constants vary when the amount of amboceptor is varied. The result is that n is always the same, but that the quantity of complement x required for complete lysis apparently becomes greater as the amount of amboceptor becomes less. The behavior of the constant k is rather similar; as the quantity of the sensitizing agent is decreased, k decreases toward zero.

This relation between the quantity of amboceptor used to sensitize the cells and the amount of complement required to give complete hemolysis is quite familiar in a general sort of way, for it has long been known that it is possible to decrease the amount of complement in a hemolytic system if the amount of amboceptor is increased (Scheller, 1910; Thiele and Embelton, 1914; Hyde and Parsons, 1927). Thus one often sees references to the "reciprocal" or "interchangeable" action of complement and amboceptor "a decrease in the concentration of either component being compensated for by an increase in the other" (Eagle and Brewer, 1929).

As soon as we try to investigate this more closely, we meet with the difficulty that the properties of amboceptor-complement systems depend rather on the quantity of sensitizing agent which is actually taken up, or absorbed, by the cells than on the quantity one introduces into the system.

It is fortunately easy to show that the absorption is rapid and that the quantity of sensitizing agent absorbed is a virtually constant fraction of that initially added, and the same appears to be true of complement, which also is absorbed at the cell surfaces provided these are first sensitized. This latter aspect of the problem has been very fully investigated by Eagle (1929) and by Eagle and Brewer (1929), who have shown that the sensitization of cells by amboceptor confers on the cells the property of absorbing complement from solution, and that the absorption is a necessary preliminary to lysis. They have shown,

moreover, that the absorption of complement is great when sensitization is produced by large amounts of amboceptor, and small when sensitization is produced by small amounts of amboceptor, and that the velocity of lysis is determined by the quantity of complement so absorbed: "With more amboceptor a greater proportion of complement is absorbed by the cell: with more complement, a smaller proportion, but a larger absolute amount. The result is the same: more complement is absorbed, and there is a corresponding acceleration of lysis." This idea, expressed in quantitative terms, constitutes the whole solution of the problem of the kinetics of hemolysis in amboceptor-complement systems.

By adding known quantities of complement to sensitized cells, throwing down the cells after various intervals with a very fast centrifuge, and titrating the amount of complement present in the supernatant fluid, the amount absorbed by the cells can be measured, and it is clear that the greater the quantity of amboceptor used for sensitising, the greater is the fraction of the initial amount of complement which is absorbed. If we sensitise the cells with amboceptor diluted 1 in 50, for instance, about half of the complement subsequently added is absorbed, whereas if we use less amboceptor, e.g., 1 in 200, the fraction of the complement which is absorbed on the cells may be only about one-tenth. Thus if we call the fraction absorbed ζ, the more amboceptor used, the greater ζ, e.g., 0.5 when the amboceptor is 1 in 50, but only 0.1 when it is 1 in 200.

The reason why there are different time-dilution curves for each different quantity of sensitizing agent will now be apparent. Suppose, as an illustration, that the quantity of amboceptor is great: then ζ will be large, e.g., 0.5. If we add 0.01 cc. of complement, half of it will be absorbed, and the quantity at the cell surfaces will be 0.005 cc. It is upon this quantity that the course and velocity of the lytic reaction depends, for a lytic reaction can take place only in the region of the cell surfaces. Next let us use a smaller amount of amboceptor, and let ζ now be 0.1. Then if we add 0.05 cc. of complement, one-tenth will be absorbed, i.e., 0.005 cc., and so the quantity of complement present at the cell surfaces will be the same as before. Two initially different quantities of complement, acting on cells sensitized with different amounts of amboceptor, thus result in the same concentration of complement at the cell surfaces, and so initially different time-dilution curves for systems sensitized with different amounts of amboceptor can be reduced to the same time-dilution curve if we consider

not the amount of complement in the system as a whole, but the amount present in the neighborhood of the cell surfaces. Quantitative measurements actually show that all the different curves corresponding to different amounts of sensitizing agent, with their different values for x, merge into one single curve when the values of ζ are taken into account, and this single curve is the time-dilution curve for complement acting as a simple hemolysin, the dilutions concerned being those which really exist in the region in which the reaction is occurring. The lysis of a given number of sensitized cells therefore requires the same quantity of the lysin complement, irrespective of the amount of sensitizing agent used, and in this respect lysis by complement is accordingly no different from lysis by simple hemolysins.

2. *Colloidal silicic acid-complement systems.* The hemolytic systems in which colloidal silicic acid acts as a sensitizing agent and complement as a lysin have much in common with amboceptor-complement systems, but are more complicated because they present such marked "hemolytic zones." By this is meant that lysis occurs only when the systems contain certain definite quantities of silicic acid and complement, whereas if either component is present in excess or is present in too small quantity no lysis occurs at all.

The kinetics of systems containing complement and colloidal silicic acid are as yet unsatisfactorily solved, but certain general properties have been established (Ponder, 1933b).

If one plots the time required for various dilutions of complement to produce complete hemolysis of cells sensitized with any particular amount of colloidal silicic acid, one obtains a time-dilution curve which shows a "zone" instead of being of the ordinary form. Small amounts of complement take very long times, and as the quantity of complement is increased, the time for lysis becomes shorter. An increase in the amount of complement beyond a certain quantity, however, results in the time for lysis becoming longer again, and finally, as the amount of complement is steadily increased, we reach a point where there is no hemolysis at all. The time-dilution curve accordingly has two asymptotes corresponding to hemolysis in infinite time, one, the upper asymptote, corresponding to a small amount (high dilution) of complement, and the other, the lower asymptote, corresponding to a relatively large amount of complement: in between these two the curve passes to a minimum corresponding to some particular dilution of complement which produces hemolysis in a shorter time than any other.

If the quantity of sensitizing agent is varied, the general shape of

these anomalous time-dilution curves does not change, but there are differences in the position of the upper and lower asymptotes, and also in the time for complete hemolysis which corresponds to the minimum between them. The position of the asymptotes, and the time for lysis at the minimum, vary with the quantity of sensitizing agent in a curious way: the latter, for instance, at first becomes progressively shorter as the amount of colloidal silicic acid is increased, and then, after passing through a minimum, becomes longer again. In this way systems containing very large or very small amounts of sensitizing agent are less readily hemolysed by the same amount of complement than are systems containing some particular intermediate amount of colloidal silicic acid, just as some particular intermediate amount of complement produces lysis more readily in systems containing a constant amount of colloidal silicic acid than do larger or smaller quantities.

Such complex relations make the solution of the kinetics in these systems exceedingly difficult, but the situation is somewhat simplified by the fact that the sensitizing agent is partly absorbed by the cells, which can be thrown down and re-suspended in saline free from the unabsorbed silicic acid. If these sensitized cells are acted upon by various dilutions of complement, a comparatively "normal" time-dilution curve results, and the zones which are observed when free colloidal silicic acid is present in the system are absent. Complement acting on such sensitized cells accordingly behaves as a simple hemolysin, just as in amboceptor-complement systems. The presence of the zones in the systems in which unabsorbed silicic acid is present, appears to be due to one or other component of the system, complement or colloidal silicic acid, being present in excess, and entering into a side-reaction with the other component.

3. *Brilliant green-serum systems.* In these systems, first described by Browning and Mackie (1914), the cells are sensitized with brilliant green or any one of the tri-phenylmethane dyes, and the lysis is brought about by the subsequent addition of the animal's own plasma or serum. The sensitizing agent and the lysin are non-specific, but, as in most complex systems, the result is dependent on the order in which the components are brought together. The addition of serum or plasma to the sensitized cells results in lysis, but the addition of the dye to cells suspended in saline containing serum or plasma results in no lysis at all. The serum component responsible for the lysis is a protein, and the different protein fractions of the plasma all produce an effect. The hemolysis is not dependent on the presence of complement; not

all proteins, on the other hand, are able to effect it, for egg albumin and gelatin are inert. The kinetics of hemolysis in these systems are similar to those in amboceptor-complement systems in that a typical time-dilution curve can be obtained by adding various dilutions of serum or plasma to cells sensitized with brilliant green, but similar to colloidal silicic-acid-complement systems in that there is one particular quantity of dye which produces optimal sensitization, either more or less dye giving less sensitization.

4. *Taurocholate-cell-serum systems.* Sodium taurocholate in a dilution of about 1 in 5000 produces lysis of human red cells comparatively slowly, say in about 10 minutes. If a small amount of serum (0.1 cc.) is added to the cells before the addition of the bile salt, or to the bile salt before the addition of the cells, almost complete inhibition of hemolysis occurs. If on the other hand the bile salt is added to the cell suspension and allowed to remain in contact with it for a few seconds, and the same quantity of serum added thereafter, there may be almost instantaneous hemolysis. This spectacular effect of the addition of the serum is similar for all types of mammalian red cells, and plasma or solutions of serum albumin, serum globulin, or hemoglobin can bring about the same effect. A similar acceleration also occurs in systems in which the lysin is sodium glycocholate, sodium oleate, or potassium oleate, and to a lesser extent if the lysin is a stearate or palmitate, always provided that the lysin is added to the cells first, and the serum (or plasma, etc.,) added subsequently.

The most satisfactory explanation for the rapidly lytic effect of the added serum is that the cells become "sensitized" by the taurocholate during the period before the addition of the serum, and that the latter then acts on the "sensitized" material as a hemolysin (Ponder and Gordon, 1935). Regarded in this way, these systems are somewhat similar to brilliant green-serum systems; in each, a substance which is a hemolysin when used in high concentration "sensitizes" the cells when used in lower concentration, and the result of the sensitization is that normal serum or plasma is able to act on the "sensitized" component as a lysin. The only difference between the two systems, indeed, is that taurocholate, acting alone, is a much more active hemolysin than is brilliant green; the difference, however, seems to be one of degree only, for it is a general rule that most, if not all, sensitizing agents are themselves lysins if used in sufficient concentration.

IV. The mechanism of hemolysis. Principally owing to the introduction of electrical measurements, there is now very little doubt that

the phenomenon of hemolysis is due either to the stretching, and possibly the mechanical rupture, of the cell membrane in the case of osmotic hemolysis, and to the chemical transformation and final breaking up of the membrane in the case of the chemical lysins (saponin, bile salts, complement and amboceptor, etc.). Just what this chemical transformation may be is not certain, and it may be different in different cases, e.g., an oxidation in the case of the irradiated dyes such as eosin (Blum, 1930a, b), a coagulation in the case of heat, a reaction with proteins in the case of saponin and the bile salts, an enzyme reaction in the case of the bacterial lysins, or a reaction with lecithin in the case of the lecithinases of venom. In all cases, however, the surface membrane is ultimately partly or wholly destroyed, and in those cases in which the lysin is a chemical substance it can be shown that some of it disappears at the same time.

In the case of most of the chemical forms of lysis, the actual hemolysis is preceded by a change in the shape of the cell, for shortly before lysis each cell loses its biconcave shape and becomes spherical without any apparent change in volume. The structure or molecular arrangement which is responsible for the discoidal form gives way rather suddenly, and the cell shortly afterwards fades from view. In some cases the ghosts persist, while in others they become either broken up or invisible because of there being insufficient refractive index difference between them and their surroundings (Millar, 1925b; Plattner and Hintner, 1930).

Considerably more information can be obtained from measurements of conductivity and capacity. As hemolysis proceeds in the case of all the chemical lysins whose action has been investigated, the conductivity of the system becomes steadily smaller owing to the resistance of the medium surrounding the cells being increased by the liberated hemoglobin. If one calculates the volume occupied by the cells themselves, as one can by treating them as non-conductors and using Maxwell's equation (Fricke and Curtis, 1935), one obtains the rather surprising result that although the cells have hemolysed, their resistance is virtually as great as before, at least up to the stage of complete hemolysis. As will be mentioned below, there are two possible explanations for this, the first that lysis takes place because the cell membrane gives way at one or two localized points ("holes"), and the second that the permeability of the cell increases at the moment of lysis, but that a "repair" of the surface, with resultant restoration of at least the greater part of the impermeability, occurs immediately after-

wards. As the system is allowed to stand after lysis is complete, however, the resistance falls to a value indicating the complete destruction of all non-conducting cell membranes. This corresponds to complete stromatolysis, and can be produced rapidly by using large concentrations of a powerful lysin.

Simultaneous measurements of capacity show that the capacity per μ^2 of cell membrane remains almost unchanged during the lytic process and subsequent stromatolysis, but the capacity of the entire system falls to zero at the end of stromatolysis owing to the continuous destruction of individual cells. The frequency dependence of the capacity at low frequencies, however, undergoes an early and steadily increasing change and it is possible that this change, indicating a change in permeability, or an injured condition, of the cell membrane, precedes all others.

In the case of osmotic hemolysis the same sort of changes are observed, except that there is no stromatolysis with its accompanying fall in resistance and capacity. When completely hemolysed, the cells appear to be as non-conductive as before, and, although they may be swollen, their capacity per μ^2 is unchanged. The only evidence of injury in the case of hemolysed cells is again found in the altered frequency dependence.

The fact that a hemolysed cell is not completely permeable, but almost if not quite as poor a conductor as a normal cell, is one which at first sight is difficult to explain. Taken alone, the observation is compatible with either the appearance of "holes" in the membrane, or with a repair of the surface film after the escape of hemoglobin, and Fricke (1934) has calculated a, the size of hole, and b, the permeability of the membrane, which require to be postulated in order to account for the measured rate of escape of pigment from the single cell (Ponder, 1934d). The measured values are compatible with either hypothesis, and one's choice largely depends on one's preconceived idea regarding the nature of surface films. The fact that hemolysed cells show an altered frequency dependence of the capacity, however, indicates that there is some injury to the cell membrane in addition to the "holes," or alternatively that the occurrence of temporary permeability and subsequent repair results in a surface film which has new properties. At the moment, the simplest hypothesis is a combination of the two, i.e., that "holes" of sub-microscopic size occur in an injured membrane, and that the subsequent stromatolysis is an extension either of the "holes," or of the injury, or more likely of both.

There is, in fact, a disquieting lack of variety in what happens to the red cell membrane during hemolysis and subsequently, as well as in what happens to the various lysins, all of which seem to be used up in combining with some component of the cell in the same general way. This lack of variety is perhaps responsible for the similarity between the kinetics of hemolysis in apparently quite different hemolytic systems, and suggests that a single process, however brought about, may be the essential one for the production of lysis. It is not difficult to imagine that such a result would be occasioned by the lysis being an all-or-none occurrence associated with the breakdown of the surface film upon which the permeability of the cell depends, for the film is, after all, a layer of only a few molecules thick, and not likely to be invested with any great diversity of properties.

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MEMBRANE PERMEABILITY TO SOLUTES IN ITS RELATIONS TO CELLULAR PHYSIOLOGY

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In every organism we are dealing with a solid-liquid system composed of different phases; therefore, every organism has structure. The main phase is an aqueous solution, divided by the constituents of the structure into a smaller or larger number of spaces. In the organism dissolved substances move in different directions and thus must pass phase boundaries; it is therefore necessary to investigate which laws govern these movements.

Frequently the non-aqueous phases form thin layers (commonly designated as membranes) between two solutions. The great majority of observers hold the view that the protoplasmic body of the cell is surrounded by a membrane, the plasma membrane, contiguous on its outer side with the watery medium. Since this plasma membrane separates living and non-living material, it is obviously of special interest to know how entrance and exit of substances across this membrane takes place, or, in physiological terminology, how cell absorption and cell excretion are realized.

It is the chief object of this article to review briefly the extensive field of physico-chemical studies concerned with the permeability of inanimate membranes to solutes, and to explore the extent to which the behavior of these inanimate systems seems comparable to that of membranes evolved by nature in living cells. Such an attempt is worth while since numerous experiences of the last decades have afforded evidence that, in the unsettled field of physiological permeability, much can be learned from handling different kinds of simple models; this is particularly true because in successfully describing the physiological processes of permeation in terms of physics and chemistry unforeseen numbers of different materials, properties and forces must be taken into account.

Now, in attempting to answer the question as to how a substance dissolved in water can be transported across a membrane into another aqueous phase, we are faced mainly with two sorts of conditions.

Either the membrane consists of a homogeneous layer of a water-insoluble substance, or it has a sieve-like structure similar to a filter. In the first case the permeability is due to the solubility of the permeating substance in the material of the membrane, in the second case diffusion takes place across the holes which directly join the aqueous phases on both sides of the membrane. However, these two kinds of penetration are by no means dissimilar. Membranes with pores narrow enough to prevent transportation of colloidal particles (ultra filters) have been known for some time. Furthermore, through the investigations of Michaelis and Collander, we are familiar with molecular sieve membranes, i.e., where the permeability is limited by a definite diameter of the pores. Such pores are so small that even individual molecules of certain substances are retained by virtue of their size. Here we are faced with the interesting question, whether or not such membranes should be considered as homogeneous solvents as well as porous filters. For even in a membrane consisting of a liquid layer the penetrating molecules have to pass through holes, created by the usual intermolecular spaces. In general we distinguish between penetration through a homogeneous solvent layer and diffusion across a membrane insofar as the intermolecular spaces in a liquid are variable, dissolved molecules squeezing themselves between the removable molecules of the solvent, whereas the existence of permanent holes with a distinct diameter seems compatible only with the conception of a solid body. The very physiological conditions might compel us to refrain from drawing such a sharp distinction between solid and liquid. Fairly sound reasons have been provided for believing that in some types of cells the thickness of the plasma membrane is no more than that of a surface film, built up of only one or several layers of molecules. From the work of Langmuir and Harkins, such films are comparable on one hand to a solid substance with a rigid porous structure insofar as the molecules are attached to each other by intermolecular forces, sometimes with such regular spacing and orientation that the structure resembles a crystal lattice, and, on the other hand, to a liquid, if under special conditions the molecules are movable—partially or in toto—so that the interspaces change.

In the light of the foregoing statements, the subject matter of this review can be reduced to three considerations, namely: to describe the properties of 1, *sieve-like membranes*, 2, *solvent-like membranes*, and 3, *surface films*, and further to point out briefly how far the comparison between inanimate systems and the physiological objects has been, or might be, successful.

At the outset, one must remember that very often physiological permeation is due not only to some sort of diffusion, but, in addition, to chemical and electro-chemical reactions in a highly complicated system, so that occasionally we may feel induced to apply the term permeation even to an act of the working machinery in a living organism.

1. SIEVE-LIKE MEMBRANES. Sieve-like membranes exist in innumerable gradations of porosity, ranging from coarse wire screening used for separating sand from stones unto molecular sieves, where even ordinary molecules are retained, when the diameter of the pores is extremely small. In this first chapter we shall discuss the permeability of molecular sieves because this type only is involved in the penetration of cell surfaces.

Dependent on the kind and preparation of the building material, membranes have either a rigid structure or a non-rigid or variable one, as, for instance, in animal skins, where shrinking or swelling of the hydrophilic colloidal components affects the interstices between the micellae. Naturally, such variability in the conditions for diffusion might be more appropriately comparable to many physiological processes where alternating activity and rest seem to accompany remarkable changes in permeability than to the constant behavior of rigid membranes. Thus, our interest in artificial membranes as models for the natural might bring us to consider first those membranes of variable porosity. But it seems more suitable to begin this review with the study of the rigid membrane, not only because they seem to be the simpler systems, but also because the striking feature of semi-permeability, or rather, selective permeability, to certain molecules and ions has been investigated with such surprising success by this method.

There can be no doubt that semi-permeability or selective permeability to molecules is exhibited by the mechanical structure of a porous solid substance. Bigelow and Bartell (1909) and Bigelow and Robinson (1918) have stated that a membrane formed out of powdered porcelain, silic oxide or graphite and more or less compressed and tightened by suitable precipitations of such substances as $BaSO_4$, $PbCO_3$ or CuS shows different degrees of permeability or even semi-permeability, when separating water from the solution of a substance with a rather high molecular size, as, for example, saccharose. Further, microscopic investigations of the structure of different precipitation membranes by Tinker (1912) have revealed the fact that these membranes are built up of distinct particles with diameters of 0.1 to 1μ, and that these particles are subdivided into still smaller ones of ultramicroscopic size, separated from each other by thin liquid sheets. But long before these authors M. Traube (1867) was already audacious enough to discuss, in connection with precipitation membranes, the existence of a molecular porosity, as concerned with semi-permeability.

a. *Permeability to non-electrolytes. The copperferrocyanide membrane.* After many more or less sporadic observations, the first systematic examination of the permeability of precipitation membranes was performed by Collander as late as 1924. It is evident from his experiments that, in general, the speed of permeation decreases with

rising molecular weight, or, better, with rising molecular volume. This result is to be expected, supposing the permeation to be due to diffusion. The molecular volumes (MV) have been calculated either from the known specific volumes at the boiling point or by applying the empirical formula of Kopp (Nernst). According to Collander, it might be expected that no satisfactory correlation between the MV and the diffusion rates could be found since the MV values refer to the pure substances, whereas the substances dissolved in water might form aggregates by polymerization or be surrounded by a shell of water molecules, increasing the space required. It is also possible that adsorption affinities between the membrane material and the solutes might have interfered with the driving osmotic forces. But, apparently, this is not true, so that it must be concluded from the experiments that the copperferrocyanide membranes behave, indeed, like inert molecular sieves. The limiting diameter of the pores of these sieves was calculated by Collander (1924) to be about 0.4 $\mu\mu$ (nearly equal to the diameter of aceton or ethylacetate).

The collodion membrane. The properties of molecular sieves can best be studied by means of the collodion membrane. When a collodion film, formed by incomplete evaporation of the solvent (alcohol-ether), is immersed in water, its permeability will be governed by the amount of solvent still present within it. Such a membrane is more or less suitable for the passage of larger molecules, or even colloid particles. However, when the solvent is allowed to evaporate completely, a membrane with the desired properties results which will maintain its behavior for a long time. Such dry membranes reveal properties characteristically different from those with larger pores, insofar as the values of the *ratios* of the diffusion coefficients of different dissolved substances are increased remarkably over those found owing to the passage through relatively large pores or to free diffusion. (This follows from tables 1 and 2.)

There have been done chiefly two sets of experiments, concerned with the permeation of non-electrolytes, one by Collander and one by Fujita and Michaelis.

In the experiments of Collander (1926) the results have been obtained by utilizing three sorts (I–III) of membranes with different pore size; membrane I with the lowest, III with the highest size. MW is the molecular weight, MR_D the molecular refraction as a measure for the molecular volume MV and calculated either from the specific refraction of the substances or by summing up their atomic refractions (both at

the wave-length D) (Nernst, 1926). (The MV calculated with the empirical formula of Kopp is about five times the MR_D-value.) The permeation of ammonia across the membrane with highest porosity is designed arbitrarily as 100.

It can easily be gathered from the table that the molecular volume is a fairly appropriate scale for the permeability. There are only a few values at variance with this result and these will be discussed later.

TABLE 1

Permeability of collodion membranes to organic non-electrolytes (R. Collander, 1926)

SUBSTANCES	RELATIVE PERMEABILITY OF MEMBRANES WITH DIFFERENT PORE SIZE			M.W.	MR_D
	I	II	III		
Ammonia	25.0	75.0	100.0	17.0	5.78
Hydrogen peroxide	10.5			34.0	5.92
Methyl alcohol	8.2			32.0	8.34
Formamide	6.0			45.0	10.61
Ethyl alcohol	1.6			46.1	12.78
Acetic acid	1.8	20.1		60.0	13.02
Urea	1.0			60.1	13.67
Ethylene glycol	0.5			62.1	14.40
Glycolic acid	0.6	10.8	39.4	76.0	14.50
Propionic acid	1.1	15.4		74.1	19.55
Monochloracetic acid	1.4	18.8		94.5	17.84
Lactic acid	0.2	4.3	30.3	90.1	19.19
i-Butyl alcohol	ca 0.2			74.1	22.19
i-Amyl alcohol	ca 0.1			88.1	26.74
Valerianic acid	ca 0.1	5.2		102.1	26.85
Phenol	3.2			94.1	27.95
i-Valeramide	0.0			101.1	28.72
m-Nitrophenol	0.7			139.1	34.03
Mannite	0.0			182.1	39.06
Quininic acid	0.0	0.2	5.6	192.1	39.96
Antipyrin	0.0			188.1	55.37
Saccharose	0.0		1.6	342.1	70.35

The experiments of Fujita and Michaelis (1926) were arranged in such a manner that $\frac{m}{100}$ urea as controlling substance, together with $\frac{m}{100}$ of another non-electrolyte passed across the collodion membranes simultaneously. The authors utilized two sorts of membranes, one immersed in water some time before complete evaporation of the solvent and thus provided with wider pores, the other completely dried and therefore operating as a molecular sieve. Table 2 gives the results.

These results seem to be in distinct disagreement with the corresonding results of Collander; whereas in his experiments the order of permeation of the different substances was in fairly good agreement with the MR_D values, in the experiments of Fujita and Michaelis this parallelism in several cases (alcohols, aceton) is missing. On the other hand, however, we find that in both kinds of membranes of Fujita, the order of permeation is nearly identical. This is significant in the following direction: Under comparable conditions (concentration gradient, time, temperature, membrane conditions) the amounts of substances passing through a membrane with large pores differ from each other in the same ratio as they do in free diffusion, and, in general, diffusion decreases inversely as the molecular size. Therefore, the agreement in the order of substances permeating across the collo-

TABLE 2

Permeability of collodion membranes to organic non-electrolytes (Fujita, 1926)

SUBSTANCES	RELATIVE PERMEABILITY OF MEMBRANES WITH		M.W.	MR_D
	Narrow pores	Wide pores		
Methyl alcohol	9.24	1.22	32	8.2
Acetone	7.08	1.11	58	16.15
Formamide	4.11	1.06	45	10.6
Ethyl alcohol	2.98	1.15	46	12.78
Propyl alcohol	1.03	1.00	60	17.52
Urea	1.00	1.00	60	13.67
Butyl alcohol	0.82	0.85	74	22.19
Ethylen glycol	0.27	0.80	62	14.4
Glycerin	0.22	0.81	92	20.63
Chloralhydrate	0.107	0.81	165	26.49
Monacetine	0.08		134	29.99
α-Monochlorhydrine	0.07	0.70	110	23.97
Glucose	0	0.54	180	37.54
Fructose	0		180	37.54
Mannite	0		182	39.06
Saccharose	0		342	70.35

dion membrane in both series of experiments provides evidence that the dried collodion membrane functions as a diffusion membrane. When, in the experiments of Collander, the diffusion rates agree much better with the M V values than in the experiments of Fujita and Michaelis this might be attributed to the part, that several brands of collodion are well known to swell in solutions of alcohols and of aceton so that the pore size might be increased.

In comparing the passage through the *wide and narrow pores* it becomes at once evident from the foregoing that the speeds of penetration through wide pores vary only slightly, even when the molecular weight or size of the molecules varies greatly, while in the case of the dried

collodion membranes much larger differences in mobilities are encountered. Small differences in diffusion are also found in the case of free diffusion, diffusion coefficient ratios as large as 1:2 being considered very large. What is the reason for the great differences in the case of dried membranes? From table 1 we learn that the differences increase as the pore size decreases (vide membrane III and I). For instance, the ratio of diffusion of ammonia and lactic acid amounts in membrane I to 125:1, in membrane II to 18:1, in membrane III to 3.3:1. The pores of membrane I are so narrow that they are impervious to all molecules with a volume corresponding to a MR_D value higher than about 24. For membrane II the limiting MR_D value is around 40. Obviously, both membranes I and II display the character of molecular sieves.

In attempting to explain the behavior, two possibilities have been advanced (Weech and Michaelis, 1928; Fujita, Collander, 1926). Either the great difference in diffusion rates may be attributed to increased frictional resistance between the larger molecules and the pore walls or to the fact that the membranes contain an assortment of pores with varying diameter, so that only the widest of the pores can serve for the passage of large molecules, while many more pores can be utilized by smaller molecules. Apparently the first hypothesis is not in agreement with the facts. In experiments designed to determine whether or not the rate of diffusion of aceton could be influenced by the presence of glycerine (aceton diffusing 62 times as rapidly as glycerine through the particular membrane) Weech and Michaelis have shown that no such effect could be demonstrated. On the other hand, the second hypothesis explains the observation that substances pass the dried collodion membrane over a rather wide range of molecular sizes, but with very different speed. The reason is that for larger molecules, like glycerine, only a small number of pores is available, i.e., a small area of the total membrane, while in the case of smaller molecules like aceton a considerably greater pore area can be entered since it is reasonable to assume that, as the size of the pore diameter increases, the number of pores greatly decreases.

As a matter of fact, nature has produced membranes with pores of thoroughly uniform diameter, the influence of which is quite different from that of the dried collodion membrane. O. Schmidt (1928) has investigated the absorption of gases by several porous minerals after having removed the imbibed water. One of these, especially, chabasit, is distinguished by a very regular structure, i.e., spacings between molecules are identical, as we know it to be in many crystal

lattices. In membranes of this kind one finds that, with increasing molecular size of the gases, absorption forces are effective only up to the volume of ethylene (M V = 44), the amount of butylene (88) and butadiene (90) absorbed being not more than 2 per cent of the theoretical value. Butylalcohol (102), ether (107), benzene (96) fail completely to enter.

There is still another interesting observation in favor of the assumption that collodion membranes contain pores of many different diameters. In experiments with glucose, Weech and Michaelis were faced with the phenomenon that in some membranes the rate at which glucose permeated slowed down after the first days or hours to a lower constant value. In comparison with the pore sizes of a dried collodion membrane, the MV of glucose is a fairly high one, so that we have to assume that only a small percentage of pores present is available to its diffusion; the retardation of the transport might be explained by supposing that gradually, as the diffusion continued, some of the *permeable pores became clogged* by the sugar molecules. Finally, the constant slow diffusion rate is maintained by the pores with a slightly larger diameter. Such a clogging effect is also manifested in collodion membranes in the presence of some narcotizing substances.

Narcotics in general are characterized by a relatively high degree of surface activity. Therefore, one can assume that they have a special ability to narrow the pore channels of a membrane by adsorptive fixation on their walls. As a matter of fact, Anselmino (1928) has found that in the presence of narcotics, like urethanes, which are shown to be adsorbed on collodion, the penetration of substances like glucose or sodium thiocyanate is more or less prevented in case the membrane has otherwise a suitable pore diameter. It is thus possible to imitate certain types of cell narcoses.

It must be admitted that quantitatively the permeability of artificial porous membranes seems very far from being comparable to the physiological because the permeation through the dried collodion membrane proceeds with extreme slowness, while the needs of the living cells are satisfied only by a relatively speedy exchange across the plasma membrane. Unquestionably, this difference is partially due to some mechanical conditions, primarily *the thickness of the artificial membranes.* According to measurements of Weech and Michaelis the thickness of the commonly used collodion membranes is approximately equal to 0.1 mm. Direct measurements of the thickness of the very delicate plasma membrane are at present impossible. But Fricke (1926) and McClendon (1926) have described methods of approaching the problem indirectly. Both of them have estimated the thickness of the red

blood cell membranes from measurements of the electric capacity made with high frequency currents. Assuming for the dielectric constant of the membranes the rather low value of 3—which is probably correct—Fricke calculated the thickness as 3.3×10^{-7} cm.; McClendon, applying a somewhat different sort of measurement, evaluated the thickness as 3×10^{-8} and as 3×10^{-7} on the basis of dielectric constants respectively 3 and 10. These measurements indicate that the plasma membrane approaches the dimensions of a monomolecular film (see p. 91). If we contrast the two values, 3×7^{-10} for plasma and 1×10^{-2} for collodion membranes it can be determined that the collodion membrane as thin as a plasma membrane would have a permeation rate 30,000 times greater than the experimentally observed one, or, in terms of time, the relationship between transport of unit amount of substance through thick and thin membranes would be one of hours to seconds.

The collodion membrane as a solvent. Hitherto we have recorded a number of permeation experiments on membranes which have been in favor of the existence of a sieve-like structure. But there are other observations, apparently at variance with this concept. In his studies on collodion membranes, Collander had to take into account some discrepancies between the diffusion rate and the molecular volume of the solutes. From table 1, it can be seen that particularly phenol and m-nitrophenol, besides propionic and monochloracetic acid, have been transported through the collodion membrane at higher speeds than could be expected from their molecular size. Fujita has encountered similar complications with ammonia. Both of these authors have considered whether the discrepancies might not be looked upon as an indication that the questionable substances diffuse through the material of the membrane as through a homogeneous solvent instead of as through the water-filled channels of the porous membrane.

This alternative view has been tested fairly extensively by Northrop (1928, 1929) in the following briefly summarized experiments: 1. Hydrogen under a measured pressure was allowed to diffuse either through a collodion membrane dried over sulphuric acid, or through the same membrane wet from water. Since the rate of diffusion of gases in water is about 1/10,000 the rate of diffusion of gases in gases, it could be expected from such experiments that the difference in the amount of gas diffusing would be very great, depending on which diffusion conditions were presented. But, within the error of the measurements the rate of passage was the same. However, the conclusion that hydrogen passes through the substance of the membrane might not be justified, since it must be considered that diffusion through the pores of a molecular sieve is not alike free diffusion or diffusion through rather wide pores, because the molecular movements are more

or less restricted in the narrow pores. 2. The gases H_2, HCl and CO_2 are allowed to pass at the same pressure through a collodion membrane. If porosity were the deciding factor, then the rate of permeation should increase as the density of the molecular volume of the gas decreases, i.e., H_2 should pass most rapidly and CO_2 most slowly. The result is that HCl passes many times more rapidly than H_2 and that CO_2 and H_2 pass at the same rate. This behavior might be accounted for by the existence of a correspondingly different solubility of these gases in the material of the membrane. 3. As a matter of fact, it can be shown that CO_2 is absorbed by collodion proportionate to the pressure and independent of the area, since the absorption is nearly the same whether equal amounts of collodion are poured into water or made into a dry membrane. Phenol, which has been noted from the experiments of Collander as permeating with an unexpectedly high speed, is also absorbed by collodion proportionate to its concentration, the partition coefficient for collodion: water being 2.8.

The rôle of adsorption. We now have to discuss still another possibility to account for rates of permeation different from those anticipated from the properties of an indifferent porous material. Söllner (1933) has suggested the following model for the pore channel of a membrane: two test tubes in vertical position are joined by a rather narrow horizontal tube near to their bottoms. This U-shaped vessel is filled with water containing lacmoid. In the upper half of the entire length of the horizontal joining tube is stretched an elongated air bubble, so that the liquid junction between the two test tubes is established only in the lower half of the horizontal tube. After addition of some drops of hydrochloric acid to one arm, it begins to diffuse very slowly along the horizontal joining tube into the other. But if butyric acid instead of HCl is used, it enters the horizontal piece and accumulates on the water air boundary and spreads out along this length with high speed, so that in a short time the acid arrives at the other test tube. This spreading phenomenon is the result of the surface activity of the fatty acid. The air in this model represents the walls in a porous substance, but can also be replaced by any water-immiscible fluid or water-insoluble solid substance manifesting an adsorption affinity. After spreading along the air bubble is complete, the surface active substance continues to enter the second test tube with increased speed, because the accumulation on the phase boundary establishes a higher diffusion gradient.

Now, the question arises whether or not adsorption plays really a rôle in the permeation of non-electrolyte solutes across a molecular sieve membrane. Naturally it does not occur in pores, the diameter of which is just large enough to enable the dissolved molecules to slip through. But since we have found that sieve membranes contain pores of different sizes, we might assume that the wider ones give rise

to an adsorptive concentration on their walls. However, the experimental evidence seems to oppose this assumption. We have learned from the study of the copperferrocyanide and the collodion membranes that the rate of permeation is rather definitely dependent on the molecular volume or runs parallel to the speed of free diffusion; it is especially noteworthy that, according to tables 1 and 2, substances known to be strongly surface active, such as butyl- or amylalcohol, do not exhibit any strikingly high degree of permeation.

From the viewpoint of physiology, it might be objected that the material of the referred membranes is basically different from that of natural membranes. For this reason, Collander (1928) has undertaken a series of diffusion experiments on *gelatine membranes* because gelatine as a protein-like substance is more related to the material of the plasma membrane. For comparison with collodion he prepared the gelatine membranes by impregnating paper vessels with a 30 per cent solution of gelatine. After drying in air, the vessels were fixed in formalin and alcohol and afterwards rinsed in water. By this procedure the gelatine was considerably deprived of its great tendency to swell or to shrink, so that the vessels took on more or less the same character of rigid porous membranes, such as dried collodion or copperferrocyanide. Diffusion experiments were performed with an assortment of solutes with a fairly high molecular size because, notwithstanding the hardening, the pore diameter of the gelatine membrane was never smaller than 0.7 $\mu\mu$ (see p. 55). In each experiment two substances, one surface active and the other surface inactive, were allowed to pass simultaneously. The substances compared had molecular sizes of roughly the same order of magnitude, for instance saccharose (MR_D 70.4) and tributyrine (76.4), glucose (37.5) and triacetine (48.7). The result was that surface activity did not appear to play any remarkable rôle by favoring the passage of the solutes.

Surface activity in these experiments means an accumulation on the air-water interfaces. Yet, we must keep in mind that many experiments (see Höber, 1926) have demonstrated that adsorption on this surface does not involve adsorption at the interface of water and any other substance. Adsorption is dependent, 1, on the existence of a difference in polarity between the two phases forming the interface, and 2, on the existence of a polar behavior of the dissolved substance (polarity meaning here different affinity in different directions). The greater the difference between the two phases with respect to polarity, the greater the surface tension between them or the smaller the mutual

solubility, and the greater the polarity of the solute, the greater is the adsorption (Langmuir, 1916, 1918; Harkins, 1917, 1920, 1926; Rehbinder, 1927; Lazarew, Lawrow and Matwejew, 1930). For example; 1, adsorption is stronger on the phase boundary between polar water and the non-polar paraffin than between water and oleic acid, both of the latter being polar, and 2, a substance like i-valeric acid with the hydrophilic COOH-"head" attached to the larger hydrocarbon-"tail" is more polar than acetic acid with the shorter "tail," and therefore adsorbed more, while non-polar substances with a symmetrical shape, like hexane or carbontetrachloride, are not adsorbed at all (see Harkins). These rules are not inconsistent with the finding that frequently adsorption from water to air goes with adsorption from water to an organic solvent (see, for instance, Traube, Weber and Guirini, 1930; King, Hall, Andrews and Cole, 1930). It is of course true that neither gelatine nor proteins in general are such solvents. But, since it has been observed in many cases that cell permeability and surface activity run parallel to each other, we must conclude that some substances like an organic solvent might be a constituent of the plasma membrane, and, indeed, for a number of reasons (see pp. 48 and 91) lipoids are assumed to play such a rôle. But it is an open question that will be discussed later (p. 48) whether, in addition to the dissolving power of the lipoids, as is claimed in the lipoid theory, surface adsorption has any significance.

We cannot leave the question as to what extent surface phenomena are involved with membrane permeation without considering *negative adsorption* as one of the factors concerned. As far back as 1877, Pfeffer pointed out that, assuming a porous structure for a precipitation membrane would not necessarily mean that the whole volume of the pore would be available for the diffusion of the solutes, but that in contrast to the frequently observed accumulation of the solute at the interface between the two phases by "positive" adsorption the pore wall could also exert attraction for the solvent molecules, so that in the fluid film, in direct contact with the pore wall, the solution might be more or less diluted. This would be "negative" adsorption. In this case only the axial thread in the pore channel would be available for free permeation of the solute; sometimes even this could be cut off if the solvent-attracting forces of the wall were sufficiently high. Negative adsorption is a fairly rare phenomenon. The evidence is based chiefly upon the measurement of the surface tension of aqueous solutions, where it has been found that—in contrast to the usually observed lowering—the tension is raised by strong electrolytes, by hexoses, disaccharides, hexites and other compounds containing OH groups. Further, inorganic salts are known to raise the surface tension at the phase boundary between water and several organic solvents (see e.g., Lórant, 1914). In all these cases the increase of surface tension apparently is due to an altered condition of the water molecules in the surface layer, caused by

stray electrical fields of solute molecules beneath the surface. Therefore, it must be admitted that in accord with Pfeffer's hypothesis negative adsorption might be one of the factors governing permeation across molecular sieves; but, on the other hand, no clear-cut demonstration of its operation can be offered up to the present (see Collander and Bärlund, 1933).

The hydration of solutes. Still another factor beside the solvent-like behavior of the membrane material and the influence of adsorption can be considered responsible for a lack of correspondence between the M V and the permeation rate, namely, the hydration of the solutes. Considerable doubt has been expressed concerning the bearing of hydration upon permeation, and only recently a fairly convincing demonstration has been supplied by Schmengler (1933).

In a number of physiological experiments it was found that the amino acids, especially glycine and alanine, enter the cells with surprising slowness, as compared with other lipoid-insoluble and surface-inactive compounds of a similar molecular size.

Schmengler tested whether or not the amino acids would show the same behavior if compared in equimolar solution with other organic compounds in diffusing across dried collodion membranes. Some of his results are detailed in the following scheme, where the figures represent molecular volumes, expressed by MR_D-values (see p. 55), and the names of substances follow the gradation of permeation velocity.

glycerine	> glycine	> erythritol
(20.63)	(16.39)	(26.77)
erythritol	> alanine	= arabinose
(26.77)	(21.01)	(31.40)
arabinose	> mannitol	> leucine
(31.40)	(39.06)	(34.87)
arabinose	> asparagine	> mannitol
(31.40)	(29.06)	(39.06)
lactamide	> α-alanine	> β-alanine
(21.01)	(21.01)	(21.01)

From these data it is evident that glycine, for example, penetrates the collodion membrane more slowly than glycerine in spite of its seemingly lower melecular size, but more rapidly than erythritol. In a similar way other amino acids also deviate exactly as they do in the physiological experiments. The following reason can be advanced: In the case of amino acids we have to deal with ampholytes, which, according to the theory of Bjerrum, exist in aqueous solution as "Zwitterions"

$+NH_3 \cdot R \cdot COO^-$, and practically entirely in this form at their isoelectric point. Such a double electric charge of opposite sign at each end of the molecule confers upon the zwitterions the character of a strong dipole, which consequently exhibits a strong pull on the surrounding water dipoles, so that a number of water molecules, instead of moving purely in random ways, orient themselves to form a more or less stable shell around the molecule thus enlarging the apparent molecular volume. In favor of this explanation, Schmengler has performed several other experiments, e.g., the last one recorded in the aforementioned scheme. In this experiment he has compared lactamide $CH_3 \cdot CHOH \cdot CO \cdot NH_2$, α-alanine $+NH_3 \cdot \underset{\displaystyle CH_3}{\overset{\displaystyle |}{CH}} \cdot COO^-$ and β-alanine $+NH_3 \cdot CH_2 \cdot CH_2 \cdot COO^-$, all of them having the same calculated molecular volume (21.01), but different permeation power, lactamide being the fastest, β-alanine the slowest. From the theoretical viewpoint this behavior is to be expected since lactamide is no ampholyte and because of the greater distance between the NH_3+ and the COO^- group, β-alanine is a stronger dipole than α-alanine. Finally, it must be remembered that besides hydration the electric charge of the dipoles might also cause the association of several such molecules into larger aggregates.

b. *Permeability of sieve membranes to ions.* When two solutions of different electrolyte concentrations are joined, diffusion takes place, and in the case of univalent cations and anions a liquid junction potential is established: $E = 0.058 \frac{u - v}{u + v} \log \frac{C_1}{C_2}$ the sign of which depends upon the relative diffusibility of the ions (Nernst); if the relative migration speed of the cation u exceeds that of the anion v, the more dilute solution is positive. In the case of potassium chloride solutions of different concentrations, no difference of potential occurs, since here u is equal to v. The same is true if we separate two potassium chloride solutions by a membrane, but only if the pores are fairly large. However, even KCl will usually give a concentration potential with material such as parchment, wax, mastic which exert a relative acceleration on the diffusion of cations. Table 3 shows the same for different salts, according to Michaelis (1926) and Fujita (1925).

Much more rarely it is observed that the diffusion of the anion is accelerated. The deciding factor is the electrical charge of the membrane substance. In most cases it is negative and therefore slows the

migration of negative ions. It is possible by artificial means to reverse the charge; under these conditions, as we shall see later, the relative mobility of the positive ions is diminished.

The effect of the charge of the membrane on the ionic migration is inversely proportional to the pore diameter; therefore, the maximum effect is obtained with the dried collodion membrane. With such membranes the value of concentration potential approaches very nearly the thermodynamical maximum equal to 58 millivolts, following from the Nernst equation, if the anion mobility approaches zero and the concentrations are 0.1 and 0.01 molar. This means that, under such conditions, we get a *selectively cation permeable membrane,* the potential difference between some two solutions with the same cation in the concentration ratio of 10:1 is here always the same, and independent of

TABLE 3

Diffusion potentials between 0.1 and 0.01 molar solutions in millivolt, + and − signs indicating the potential of the more dilute solution (L. Michaelis)

SALT	A SOLUTIONS JOINING DIRECTLY	B SOLUTIONS SEPARATED BY A PARCHMENT MEMBRANE	B − A
KCl	0	+10	+10
LiCl	−15	−4	+11
K_2SO_4	+15	+27.5	+12.5
NaOH	−32	−14	+18

the anion present (Michaelis, Collander, 1925; see further Wilh. Ostwald, 1890).

From the selective cation permeability, manifested by the electric measurements, there follows a very characteristic influence upon the diffusion processes. When a salt solution is separated from pure water by a suitable membrane, no trace of the salt can be detected in the water even after days. But, the diffusion proceeds with a remarkable speed if the pure water is replaced by another salt solution. To state more exactly, *the cation of one salt* diffuses across the membrane because it *can be exchanged for a cation of the other salt,* migrating in the opposite direction (Michaelis and Perlzweig, 1927) For instance, from HCl on one side of the membrane and KCl on the other side, H^+ and K^+ diffuse in opposite directions; with KCl on one side and KNO_3 on the other nothing happens.

Now, evidence can be provided that with the dried collodion mem-

brane there exist differences in the rate of cation permeability. Instead of placing one electrolyte in different concentrations on either side of the membrane, we separate two different electrolytes of the same concentration, and measure, instead of the *"concentration potentials,"* what Michaelis has called *"chemical potentials."* Table 4 shows the result obtained in an appropriate membrane with 0.1 molar solutions.

It follows from table 4 that when the cations on opposite sides of the membrane are different, potential differences are built up which are much greater than in free diffusion. The reason is that on the one hand the mobility of the anion in the cation-permeable membrane can be neglected, and that on the other hand the relative *mobilities of*

TABLE 4

Potential differences in millivolt between 0.1 mol solutions and relative mobility of the cations (L. Michaelis)

+ and − signs indicating the potential of the KCl solution

SALTS	MILLIVOLTS		RELATIVE MOBILITY OF CATIONS		SALTS	MILLIVOLTS WITH DRIED COLLODION MEMBRANES
	With dried collodion membranes	In free diffusion	Within a dried collodion membrane	In free solution		
KCl:HCl.....	+93	+27.3	42.5	4.9	KCl:KBr.....	+2
KCl:RbCl.....	+8	+0.58	2.8	1.04	KCl:KJ.....	0
KCl:KCl.....	0	0	1	1	KCl:K_2SO_4.....	+2
KCl:NaCl.....	−48	−4.47	0.14	0.65	KCl:$K_3Fe(CN)_6$...	0
KCl:LiCl.....	−74	−6.96	0.048	0.52	KCl:KOH.....	+2

the cations differ much more within the membrane than without, as can be calculated from the formula:

$$E = 0.058 \log \frac{u_1 + v}{u_2 + v},$$

where u_1 and u_2 are the respective mobilities of the cations and v the anion mobility equal zero. From such calculation it appears that the ratio of the mobility of hydrogen to lithium in the membrane is almost 1,000, while in free solution less than 10. Table 4 shows, further, that anions exert no influence.

Evidently the order of the atomic sizes of the alkali metals does not correspond to that for cation mobilities, but is reversed, since Li^+ with the lowest atomic weight is the slowest, Rb^+ with the highest weight

the fastest. This is explained according to the theories of Debye and Born that these ions are carriers of a shell of polarized water dipoles, Li^+ dragging along the largest shell, Rb^+ the smallest (see p. 89). Even greater differences between Li^+ and Rb^+ diffusion rates occur when contrast between free diffusion and diffusion through a membrane is considered; this is attributed to another influence already mentioned (p. 58), namely, the apparent existence of a great variety of size of the pore diameters of the membrane, with the effect that only a small number of pores is wide enough to allow the entrance to the largest ions like Li^+, whereas a more extended area of the membrane, composed of a greater number of smaller sized pores, is available to the diffusion of Rb^+.

When the cations are bi- or trivalent, the potential differences are uncertain; this seems to be due to a very low permeability of the dried collodion membrane to such ions.

The great differences in ion mobilities must play a rôle in determining the time after which the final diffusion equilibrium is attained when a cation exchange across a cation-permeable membrane occurs. Obviously *the migration rate of the slowest ion should be and is decisive.*

However, under certain conditions, this diffusion equilibrium is attained only in passing significant stages as has been worked out by Netter, 1928 (see also Brooks, 1929). In one set of his experiments a dried collodion membrane separated a solution (1) consisting of $\frac{m}{1400}$ K_2SO_4 + $\frac{m}{10}$ H_2SO_4 from the solution (2) consisting of $\frac{m}{1400}$ K_2SO_4 + glucose. The membrane was permeable to K^+ and H^+, and nearly impermeable to SO_4^{--}; by the addition of a proper amount of glucose the solutions were rendered isotonic. The tendency for H^+ to pass the membrane was determined by the steep concentration gradient $10^{-1} \rightarrow 10^{-7}$; but for electrostatic reasons such a passage was not possible except by an exchange against an equivalent amount of K^+. Notwithstanding the fact that the concentration of K^+ was the same on both sides, such an exchange did proceed and K^+ was *transported against an increasing concentration gradient.* $H^+_{(1)}$ and $K^+_{(2)}$ meet in the pore, moving in opposite directions, the chance of their meeting at the same moment being proportionate to $H^+_{(1)} \times K^+_{(2)}$; the same is true for the corresponding cations $H^+_{(2)}$ and $K^+_{(1)}$, their chance of meeting being proportionate to $H^+_{(2)} \times K^+_{(1)}$, so that the exchange in the first direction is cancelled partially by that in the second, until equilibrium is attained, i.e.,

$$H^+_{(1)} \times K^+_{(2)} = H^+_{(2)} \times K^+_{(1)} \quad \text{or} \quad \frac{H^+_{(1)}}{H^+_{(2)}} = \frac{K^+_{(1)}}{K^+_{(2)}} .$$

The experimental results are consistent with this theory. The following scheme gives one example of the experiments.

In the beginning	*After 13 days*
$\frac{K^+_{(1)}}{K^+_{(2)}} = \frac{1}{1}$	$\frac{K^+_{(1)}}{K^+_{(2)}} = \frac{>25}{1}$
$\frac{H^+_{(1)}}{H^+_{(2}} = \frac{10^{-1}}{10^{-7}} = \frac{10^6}{1}$	$\frac{H^+_{(1)}}{H^+_{,2)}} = \frac{52}{1}$

Because of the thickness and therefore very low permeability of the membrane, the experiment was allowed to run over 13 days. The chief result was that, while the concentration gradient of H^+ decreased by diffusion, a strong concentration gradient of K^+ was established. The accumulation of K^+ was due to the experimental trick that by addition of glucose the great difference of osmotic pressure between the solutions was neutralized. Without this addition the final equilibrium, i.e., equality of ion concentrations on both sides of the membrane, would have been attained mainly by osmosis instead of diffusion. Such selective cation permeability of a membrane giving rise to a transitory non-equilibrium is strongly suggestive of similar effects of ion accumulation in living cells (compare Netter, 1929, 1934; Bruch and Netter, 1930; Fenn and Cobb, 1934).

As has been mentioned before (see p. 65), the preferential cation permeability of the most membranes is due to the electro-negativity of the membrane material. This is proven by the fact that by changing the charge from negative to positive the membrane becomes anion-permeable. The easiest way to demonstrate this is to use as a membrane material an ampholytic colloid like protein or gelatine. Thus, Höber and Matsuo (1923), Mond (1924), and Michaelis and Fujita (1925) have found such membranes, when separating two solutions of the same electrolyte but of different concentrations to exert no influence on relative mobilities of cation and anion provided they were kept at the iso-electric point of the colloid-ampholyte. But when the reaction was shifted to the acid side the mobility of the anion prevailed, while on the alkaline side the mobility of the cation (Bethe and Toropoff, 1914, 1915), i.e., concentration chains, with different reactions by addition of acid or alkali, are working in opposite directions.

The effect of the charge of the membrane material is best demonstrated with the dried collodion membrance, the negative charge of which can be inverted by basic dyestuffs, by alkaloid bases or by phenylendiamine (Mond, 1928; Wilbrandt, 1935). For instance, a

collodion membrane, positively charged by rhodamine B, gave the following values.

TABLE 5

Potential differences in millivolt across a dried rhodamin B-collodion membrane between 0.1 mol solutions (Mond)

(−signs indicating the potential of the NaCl solution)

NaCl:NaSCN	−60	NaCl:NaCl	0
$NaCl:NaNO_3$	−51	NaCl:KCl	−2
NaCl:NaJ	−33	$NaCl:KNO_3$	−53
NaCl:NaBr	−20		

Obviously, table 5 is a counterpart to table 4 in demonstrating clearly the electromotive effectiveness of anions and the ineffectiveness of cations; the positively charged dried collodion membrane behaves as a *selective anion permeable membrane.*[1] However, whereas with the cation-permeable membrane the *order of potential differences* follows the order of cation mobilities in free solution, this is not true with the anion-permeable membrane in respect to the anion mobilities, since their mobility decreases in the series: I, Br > Cl > NO_3 > SCN. We shall return to this point later.

Additional evidence of selective anion-permeability is presented by the following observations: 1. In a concentration chain with a potential difference across a cation-permeable membrane, the more dilute solution is always positively charged, the opposite is true of an anion-permeable membrane. Like cation-permeable membranes, in systems with concentration ratios 10:1, the potential differences can approach the thermodynamical maximum of 58 millivolts (see p. 66). 2. As with cation-permeable membranes and cations (see p. 66), anion-permeability can be proven analytically by an exchange of anions; for instance, a rhodamine-collodion membrane separating KCl and $NaNO_3$ allows only Cl and not K to pass from the potassium chloride solution (Cl interchanging with NO_3, Mond). This is analogous to the well known demonstration of selective anion permeability in red

[1] The selective cation and selective anion permeability of the membranes is analogous to the selective cationic and selective anionic conductivity of some solid substance like the silver halides or the lead halides; e.g., in AgCl only Ag^+, in $PbCl_2$ only Cl^- is transported by the electric current (Tubandt, 1928). The reason is dissociation into simple and complex ions, e.g., $(CdI_2)_n = Cd^{++} + (Cd_{n-1}I_{2n})^-$, only the former of which is movable in the conducting solid.

blood cells, the counterpart of the selective cation permeability of muscle and nerve.

Another phenomenon that is likewise of some physiological significance is due to the different rates of permeability to cations as well as to anions in collodion membranes which are not completely dried and therefore not exclusively cation or anion-permeable. When two equimolar electrolyte solutions are separated by such a membrane and a direct current is sent through in either direction, the applied potential being the same, it appears that the resistance varies more or less with the direction of passage of the current, so that the membrane shows irreciprocal or *unilateral conductivity*, or behaves like a rectifier (Labes and Zain, 1927; Blinks, 1930). The electrolytes may be $KHPO_4$ and NaCl. When the current is directed from $KHPO_4$ to NaCl, the resistence is low, because the direction of the current is such as to carry the ions which are comparatively mobile in the membrane (K, Cl), whereas with the opposite direction the resistance is high, Na and HPO_4 having the larger ionic volume and hence the lower mobility. Therefore, these changes are mainly due to real ohmic resistance; changes of the resistance due to back electromotive forces of polarization are of minor importance. Similar conditions have been found in living cells, for instance, in Valonia, where measurements can easily be made by inserting one electrode through the protoplasmic walls into the large cell vacuole, the other being placed outside (Blinks).

The nature of the selective ion permeability of sieve membranes. Before continuing to study the properties of sieve membranes we must inquire somewhat further into the nature of the selective ion permeability (see Freundlich, 1932; Michaelis, 1926; H. Müller, 1933). In case of a collodion membrane, the membrane material is negatively charged. This means presumably that anions are rigidly attached to the surface of the pore walls by adsorption. In the immediate neighborhood of this surface the electrostatic forces are large enough to fix a number of corresponding cations; only at a greater distance from the solid surface beyond the "rigidity boundary" ("Abreiss-schicht" of Freundlich) are the cations movable, although the movement is impeded by the attractive forces of the surface. Now, if a positive ion from an electrolyte solution outside the membrane happens to approach the surface of a pore, it will be attracted to the surface; negative ions, on the other hand, will be repelled by the negatively charged surface. Consequently, we will find, in a given period of time, more positive ions near the surface than removed from it. If the pore is narrow enough, negative ions cannot enter at all, while any number of cations that happen to be in the "diffuse double layer" (Gouy) outside the rigidity boundary can pass at will. Furthermore, the greater the attractive forces between surface and cations the larger will be the surplus of

positive charges. Such attractive forces could depend on three factors: valency of ions, unsaturated valency of the atoms or van der Waals attraction of molecules.

The permeability of collodion membranes to organic ions. According to Michaelis and Mond, the cation-permeable collodion membrane is permeable to alkaloid cations, the anion-permeable membrane to thiocyanate. Both types of organic ions pass with higher speeds than could be expected from their ionic size or from their speed of migration in free solution. These facts raise the question as to the permeability of the sieve membranes to organic ions in general. Until now, this question has been studied only with anions using dried rhodamin-collodion membranes and measuring membrane potentials (Höber, 1935).

1. It has been stated that the anions of strong electrolytes like the alkali salts of the sulfonic acids, for instance, toluene-sulfonate, $CH_3C_6H_4SO_3^-$, pass much more easily than does $SO_4^=$. This can be explained as follows: the aromatic sulfonates belong to the so-called hydrotropic or *hydropolar electrolytes*; the configuration of their molecule is such that one part,—using the terminology of Adam,—the "head," has a strong affinity for water (see p. 92), whereas the other part, the "tail" is hydrophobic, i.e., through some sort of van der Waals force has an affinity for some water-insoluble material.

Properties of this kind revealed, for instance, by some observations of Freundlich and Slottmann, 1927. According to those investigators the positively charged particles of colloidal ferric hydroxide are discharged by sulfonates, the order of effectiveness being: benzene-sulfonate < p-toluene-sulfonate < p-ethyl-benzene-sulfonate; in the same order their ability to cause flocculation of ferric hydroxide and the surface activity of their solutions increases. This indicates that the hydrophobic organic tail of the negative ions is adsorbed on the surface of the colloidal micellae with correspondingly increasing strength. According to Neuberg (1916), Freundlich and Krüger (1929), Katz (1933–34), von Kuthy (1931), another type of hydropolar activity of these compounds is their ability to bring into solution many sparingly soluble substances, such as starch, gelatin, alkaloids, dyestuffs, lipoids, by attaching their hydrocarbon tail to these substances and thus conferring upon the whole complex the high hydroaffinity of the head, the sulfonic group.

These and other statements lend support to the assumption that, in contrast to the surface-inactive sulfate ion, the sulfonates are concentrated more nearly to the surface of the pores and spread along the walls (see p. 61). In this way a steeper diffusion gradient of ions across the membrane is set up and the permeability increased.

2. The same principle is sound for explaining the relatively high de-

gree of permeability encountered with a number of other organic anions. For instance, the aromatic anions, salicylate and benzoate, are strongly hydrotropic as well as the anions of the fatty acids with longer chains of C-atoms, like caprylate, nonylate, oleate, abietinate, laurinate, all of which cause membrane potentials nearly as high as nitrate. On the other hand, this hydrotropic behavior is not exhibited by anions like lactate, fumarate, tartrate, citrate. Their polar character is indistinct; they are surface-inactive (see p. 92), and the membrane potentials developed are nearly identical with that of sulfate. In the case of the higher fatty acids, another factor comes into play which serves to increase the height of the potential difference, namely, that by hydrolysis of the salts free acid is also liberated which is not only surface-active but also presumably soluble in the material of the membrane, (see p. 61). In addition, by virtue of their soap-like character, surface-active complex ions are formed (Donnan and Potts, McBain, quoted in Freundlich, 1932). All these features have a special physiological significance because we meet comparable conditions in the plasma membrane of the anion-permeable red blood cells (Höber). Finally, we must reiterate the fact (see p. 70) that the penetration rate of the inorganic anions does not follow the order of the electrolytic mobilities: J, Br > Cl > NO_3 > SCN, but the order: SCN > NO_3 > J > Br > Cl > SO_4, which is well known in physiology and in colloid chemistry as the Hofmeister series. It might be added that the Hofmeister series is also the series of adsorbability, SCN having the greatest, SO_4 the smallest surface activity. In the same order of the series is also a corresponding decrease in hydration of the ions, the shell of water dipoles conflicting with the adhesion of the ions to the collodion and other comparable substances (Freundlich and Schnell, 1928; Katz, 1933).

Compound sieve membranes. When both sides of a dried collodion membrane are in contact with the identical electrolyte solutions, one would expect to find no potential at all. But it happens not infrequently that a potential can be recorded which is due to some asymmetry in the membrane structure. Wilbrandt (1935) studied such *asymmetry potentials* artificially produced. If an ordinary cation-permeable collodion membrane is stuck to one, impregnated either with a basic dyestuff or with an alkaloid salt, and hence anion-permeable, a double membrane is obtained. Such a membrane with $\frac{m}{1,000}$ KCl on both sides yields an asymmetry potential of several hundred

millivolts. That such a membrane is cation-permeable on one side and anion-permeable on the other can be proven by the demonstration that the potential difference is affected by changing the cation on the side permeable to it, or the anion on the side permeable to it. This is shown by table 6.

According to Wilbrandt, the positivity on the cation-permeable side of the membrane is determined by the tendency of the inside quinine ions to leave the membrane on this side. Consequently, the asymmetry potential decreases, as the concentration of the electrolyte in solution increases; for instance, with $\frac{m}{1,000}$ KCl on both sides the membrane gives 251 millivolts, with $\frac{n}{100}$ only 163. The rate at which the potential is lowered with different electrolyte solutions depends on the rate of permeability to cations or to anions on both sides; for example, table 6 shows that when K_2SO_4 replaces KCl on the anion-permeable side the potential increases from 57 to 86 millivolts because the anion-permeable side of the double membrane is less permeable to SO_4 than to Cl.

TABLE 6

Chemical potentials of a double membrane with asymmetry potential (Wilbrandt)
(0.1 m solutions, + sign of potential on the cation-permeable side)

QUININE-COLLODION	COLLODION	MILLIVOLTS
KCl	KCl	+57
K_2SO_4	KCl	+86
KCl	K_2SO_4	+58
KCl	KCl	+57
KCl	NaCl	+82
NaCl	NaCl	+86

It is not unlikely that conditions comparable to those investigated by Wilbrandt are to be found in some plasma membranes. According to Osterhout (1927, 1933), Blinks (1930) and Damon (1931), a potential difference can be measured in Valonia, Halicystis or Nitella when not only the inner but also the outer surface of the protoplasm is brought into contact with the cell sap. Similarly, with Halicystis, when both surfaces are bathed with the same sea-water at pH 5 (Blinks, 1935).

Naturally, the material of these physiological membranes is completely different from that in the model experiments just described. It might, however, be imagined that in the plasma membrane of a living cell asymmetry potentials could also have their origin from conditions where two layers of different proteins of different iso-electric

points formed a membrane. One side of such a membrane would be cation-, the other anion-permeable, as long as the pH of the same electrolyte bathing both sides ranged between the respective isoelectric points. Shifting the pH on either or both sides of the membrane will result in a change of potential as well as permeability (p. 69, and Mond, 1924).

Membrane mosaics of wide and narrow pores. When the pores of a sieve membrane, separating two solutions of the same electrolyte but of different concentration, are wide enough, the potential measurable across the membrane is identical with the liquid junction potential $E = 0.058 \frac{u - v}{u + v} \log \frac{C_1}{C_2}$. Decreasing the pore diameter results in a potential increase, approaching the maximum value, $E = 0.058 \log \frac{C_1}{C_2}$.

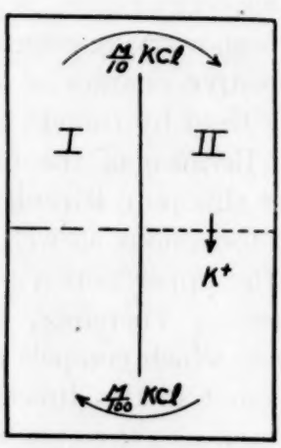

Fig. 1

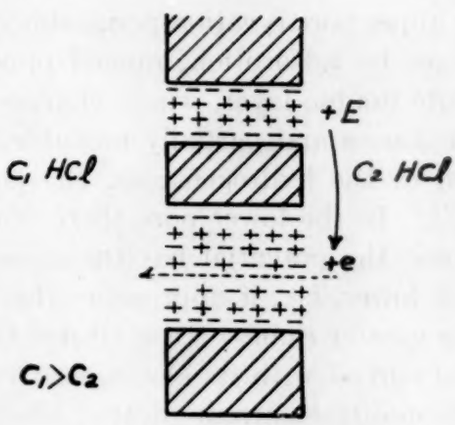

Fig. 2

This is because either v or u approaches zero, or, in other words, the membrane becomes either selectively cation- or anion-permeable. In a sieve membrane, like dried collodion, different pore diameters are coexistent and regional membrane potentials of different values should be detected by applying micro-electrodes. Therefore, according to the theory of Söllner (1930) (see further Söllner and Grollman, 1933; Bartell, 1914, 1916, 1921; T. Hamburger, 1917; J. Loeb, 1919–20), it must be assumed that the potential differences operating between different regions will bring about the flow of an electric current, the qualities of which we have now to discuss.

The scheme, figure 1, represents two areas of a collodion membrane, the left (I) showing wide pores, the right (II) narrow ones. The mem-

brane divides the vessel into two parts; the upper one is filled with $\frac{m}{10}$, the lower with $\frac{m}{100}$ KCl. Further, the vessel is partially divided by a wall into a left and a right part. Now, collodion being negatively charged, area II is selectively cation-permeable and therefore the seat of an electromotive force of approximately 58 millivolts; while in area I there will be no potential because the pores are so wide that, as in free diffusion, both K and Cl can move with identical speed. Then, an electric current will circulate in the direction of the arrows, and the electromotive force of 58 millivolts will move not only the ions but also establish an electro-osmotic flow of solution through area I in the same direction.

This behavior is illustrated even better in the scheme (after Söllner), figure 2. It portrays two pores of the sieve membrane, separating two solutions of hydrochloric acid with the concentrations c_1 and c_2 ($c_1 > c_2$). The upper pore is cation-permeable only, its pore wall carrying negative charges by adsorption, ranged opposingly to positive charges of the electric double layer, whose charges are partially fixed by the electrostatic forces and partially movable (see p. 71). Because of the high speed of the hydrogen ions, the potential across this pore is positive (+E). In the lower pore there is room enough for anions as well as cations; the potential has the same sign as in the upper pore (+e), but is lower, i.e., it approaches that of free diffusion. Therefore, +E being greater than +e constitutes the driving force which compels the liquid thread with its electropositive surface to move in the direction of the positive current (dotted arrow) and thus increases the common osmotic flow concerned with the concentration difference $c_1 - c_2$. This is what is known as *"positive anomalous osmosis."*

Even greater interest has centered on the older observation of Graham on *negative anomalous osmosis.* We shall see that the theory of Söllner serves also to explain this phenomenon. The scheme, figure 3, pictures a case of negative osmosis, which is brought about by the diffusion of LiCl from the higher concentration level c_1 to the lower c_2. In this case $v_{Cl} > u_{Li}$, the lower and wider pore becomes the seat of a negative liquid junction potential −E, while the upper pore, because of its narrowness and the negative charge on its walls, a condition favoring mobility of cations only, gives a low membrane potential +e. Therefore, the higher potential −E enforces electrosmosis in the upper pore, directed from c_1 to c_2. Finally, figure 4 shows the scheme for negative osmosis, where hydrochloric acid diffuses through a membrane with positive charge.

It follows that two factors govern the direction of the anomalous osmosis: 1, the thermodynamical membrane potential, comparable to that of a metal electrode, and 2, the electrokinetic potential, depending on the sign of the fixed charge of the double layer. This theory of Söllner has been verified by experiments. It holds true not only in the case of different concentrations of the electrolyte but also with different electrolytes. Conceivably, the process of electro-osmosis can continue only as long as the nonequilibrium, involved in the gradient of concentrations, is maintained. From the experiments of Bethe and Toropoff (1914, 1915) the electro-osmotic transport must be accompanied by opposite changes of ionic concentrations, as, for example, in the case of H ion concentration, on the two sides of the membrane.

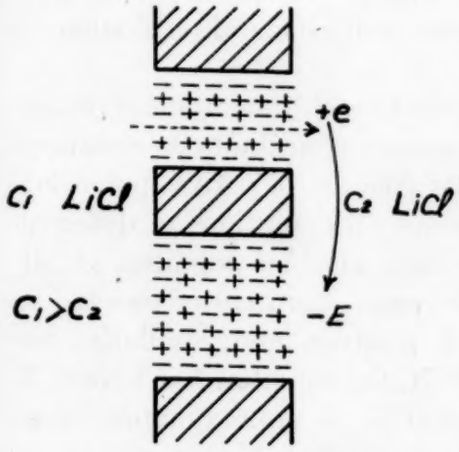

Fig. 3

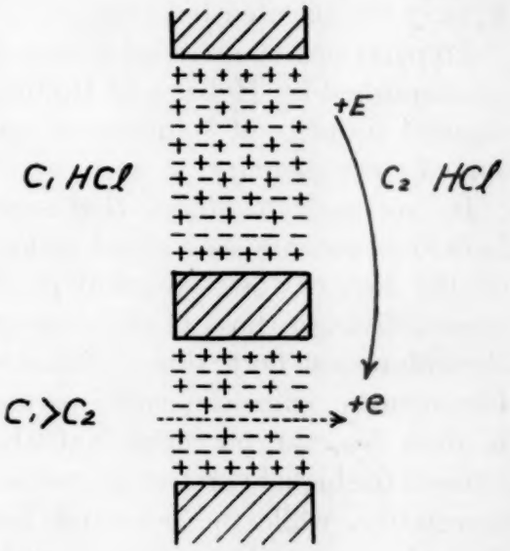

Fig. 4

It is difficult to properly evaluate the bearing of these studies on physiological investigations. Of late, the fact that such bioelectrical potentials might be utilized for electro-osmotic transport of water for different purposes has often been taken into account. After the elucidation of the physical conditions for such a phenomenon, it might be appropriate to consider some physiological conditions comparable to them. For instance, more recently evidence has been advanced not only for the existence of porosity in the plasma membrane of cells but also that probably the cementing substance itself between the cells is a sieve with large pores. Currents would thus be set up to circulate, for instance, through cells and intercellular spaces of an epithelial layer. Furthermore, the material of the cellular surface sieve is chemically

certainly more variable than collodion. Therefore, it can be assumed that metabolic reactions may increase, decrease or reverse the electric charge. A shift in H ion concentrations, due, for example, to some metabolic change, may alter the charge of colloidal ampholytes like protein, so that fluctuations in the amount or even direction of electro-osmotic transport might occur (for some calculations concerning the electric energy available from cells for the electro-osmotic transport compare with Fürth, 1933).

Membrane mosaic with cation-permeable and anion-permeable areas. Some special features of protoplasmic permeability, associated with the simultaneous movement of cations and anions across a membrane have suggested the hypothesis that there is a mosaic of regions selectively permeable either to cations or anions. Such a membrane must display a fairly complicated behavior.

Preparations of artificial mosaic membranes have been successfully accomplished by Höber and Hoffmann (1928) by allowing closely juxtaposed droplets of solutions of collodion and rhodamin-collodion to spread over mercury.

It can easily be shown that such "patch-work" membranes whose individual patches show either cation or anion-permeability as measured by the sign of their chemical potentials (see p. 67) give potentials intermediate to those of the separate areas with solutions of different electrolytes on both sides. Sometimes they give no potential at all. For instance, since the cation-permeable areas afford easier passage to K than Na, the potential NaCl-KCl is positive, and, similarly, because of the higher permeation power of SCN, the potential NaCl-NaSCN is negative, while the potential NaCl-KSCN is approximately zero, since the penetration of K as a cation is counterbalanced by the penetration of SCN as an anion.

If an electrolyte solution is separated by a mosaic membrane from distilled water, permeation might be supposed to be nil, because each of the alternating areas of the mosaic is permeable only either to the cation or to the anion. But since all areas are electrically connected by the conducting salt solution, the positive charge of the distilled water, resulting from the passage of some few cations across a cation-permeable region, will be electrically neutralized by the same number of anions moving across an anion-permeable area. This indicates that currents are circulating through the pores of the membrane by means of which electrolyte will be transported from the more concentrated to the more dilute solution (Söllner, 1932). This process is pictured by figure 5.

In a similar way, currents will circulate also when different electrolyte solutions are separated from each other by the mosaic membrane and also when the different areas are only prevailingly but not exclusively permeable either to cations or to anions. In either case, according to the theory and the experiments of Söllner, electro-osmosis may take place. Obviously, a number of different processes must operate for the final attainment of equilibrium, i.e., equality of concentrations on both sides of the membrane.

But, according to Brooks (1929), these tendencies to bring about final equilibrium may be opposed by still another process. In order to consider this let us turn again to the mosaic of selective cation- and anion-permeable regions. Taking first separately a cation-permeable area interposed, e.g., between an acid solution (i) and a neutral salt solution (o), we might expect, disregarding the aforementioned proc-

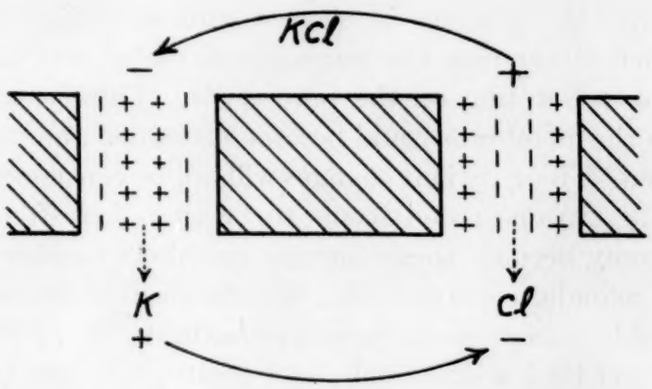

Fig. 5

esses, that hydrogen ions would move in one direction while an equivalent amount of potassium ions would pass in the opposite. According to what has been outlined previously (see p. 68), this cation exchange would go on until $\frac{H_i}{H_o} = \frac{K_i}{K_o}$, i.e., K would enter the more acid solution not only until its concentration had become identical on both sides but until its concentration ratio equalled that for H. The result would be an accumulation of K on the more acid side. Now, Brooks, taking into account in the same manner the anions in the case of anion-permeable areas of the mosaic membrane, attempts to interpret the different distribution of different ions between the cell sap of Valonia and sea-water as external medium. Such a system represents a state of nonequilibrium, maintained by the continual production of carbonic acid inside the cells, accompanied by an exchange of hydrogen ions

against cations, of bicarbonate ions against the anions of the sea-water. Since the penetration power of K is considerably higher than that of Na, Cl and HCO_3 about the same and SO_4 very slow, the theory seems to be in agreement with the analytical data which indicate a great surplus of K inside and Na outside and a small surplus of Cl inside, a very small one of SO_4 outside. However, it seems questionable whether the ionic nonequilibria arising from ionic exchange with the mosaic areas separated from each other might not be abolished immediately by the aforementioned currents in case the areas are not separated.

Sieve membranes with variable pore size. The question of changes in permeability of plasma membranes is much more frequently discussed than answered. Hence it might be useful to consider under what conditions the permeability of artificial membranes can be changed. There are several possibilities, of which chemical transformation of membrane material induced by metabolic reactions is of prime importance. Secondly, the porosity of sieve membranes may be changed by solutes which either clog the pores by virtue of the size of their molecules or by adsorption on the pore walls. Thirdly, there is the possibility that the membrane could become distended and its mesh size thereby modified. These various changes will not be considered in detail. More appropriate to discussion seems to be the *effect of swelling and shrinking*, not only because these changes are likely to play an important rôle in a colloidal substrate like the plasma membrane, but also because reversible variations of pore permeability in artificial membranes have as yet been studied only with hydrophilic gels formed into membranes.

Suitable to this treatment are chiefly gelatine and agar. There is some question about the structure of these gels as to whether they are built up of a coherent solid phase pierced by channels or knit together of threads or discontinuous in that the colloid particles swimming like islands in the liquid phase (see Freundlich, vol. 2, 1932). In any case, some kind of porosity is assumed. Now, in many earlier investigations it has been shown that a solute penetrating such a gel passed with the same speed as in free hydro-diffusion, provided the molecules are sufficiently small, whereas with larger molecules such as those of many dyestuffs or dissolved colloids the diffusion is relatively slowed or even completely stopped. This behavior is distinctly indicative of porous structure. But often the diffusion rate also will be diminished if the concentration of the gel is raised, even when the molecular volume of the solute is small (Bechhold and Ziegler, 1906; Öholm, 1913; Fried-

man, 1930; Friedman and Kraemer, 1930). This apparently means a decrease in porosity, the micellae of the colloid being packed closer together. We will come back to this point later.

There is a question whether with swelling or shrinking of such a gel changes of permeability are involved. Only few experiments have been arranged in such a manner as to give clear results. The experimental conditions in the studies of Anselmino (1928) are as follows: 1, membranes are formed from approximately 12 per cent gelatine; 2, the permeating substance is an indifferent non-electrolyte of medium size like glucose; 3, owing to the thickness of the fused membranes, the glucose solution must be brought into diffusion equilibrium with the gel before starting the experiment; 4, when swelling is produced by changing the reaction either to the acid or to the alkaline side, preliminary equilibrium must also be established with a set of HCl and NaOH solutions; 5, in fusing the membranes, different amounts of the gelatine solution must be taken corresponding to the strength of acid and alkali so that in spite of swelling the final thickness of all membranes is the same. Under these conditions the experimental results are as follows: permeability is at the minimum near the isoelectric point of gelatine, equal to pH 4.7; it rises more and more on the acid side with pH decreasing to 3.1 and on the alkaline side with pH increasing to 8.8, i.e., *swelling promotes permeability.* This conclusion is justified since glucose passes the membranes with the same speed, independent of pH changes after the swelling power of the gelatine has been prevented by treatment with formol.

According to Anselmino, the following explanation in regard to the connection between swelling and permeability may be proposed: the gelatin micellae are surrounded by a shell of water dipoles. On either side of the isoelectric point they are fixed by the positive or negative charges of the micellae, the size of the charge determining their number. Therefore, area and thickness of different membranes being equal, the number of closely packed micellae in one membrane is smaller, the larger the shell of water surrounding them. According to the theories of Debye and Born it is assumed that the water dipoles are fixed and oriented as in a solid body; farther from the charged particles they are more movable. Now, in case the fixation of water involved with swelling be strong enough, so that solutes cannot enter the shell, the permeability remains rather unaltered because with spherical micellae the intermicellar space would be quite independent of the diameter of the spheres. On the other hand, the imbibed water remaining unbound, the permeability should increase with the swelling. As a matter of fact, the permeability increases much less than the intake of water. Therefore, it may be assumed that the greater part of water is attached to the electrically charged micellae as a strongly fixed shell of dipoles, while the smaller part is movable and free for diffusion. This means increase of permeability.

In another set of experiments Anselmino has demonstrated that, with electrolyte instead of glucose, the influence of reaction change on permeability is entirely different. Experiments have been performed, e.g., with an acid dyestuff, cyanol, whose anions stain. It is a strong electrolyte, its electrolytic dissociation being independent of pH. One necessary condition for obtaining clear results is, again, that the diffusion equilibrium between gel and dyestuff solution be established before starting the experiments. Raising the pH from the isoelectric point progressively diminishes the passage of cyanol even though the alkaline reaction enlarges the pores; the reason may be that the dyestuff anions are repelled from the pore walls so that only space for free diffusion remains in the axis of the pores. On the other hand, with decreasing pH the permeation rises strikingly, perhaps not only because the whole cross-section of the pores is available for diffusion, but also because the dyestuff anions and especially their organic tails are possibly adsorbed by the pore walls (p. 72). Thus diffusion could proceed at a higher concentration gradient (p. 61). In toto it follows that here the influence of pH on the pore size is rather completely overshadowed by the effect of polar adsorption.

Regarding the connection between swelling or shrinking and permeability, it would be equally interesting to follow the *influence of neutral salts;* however, this problem has been settled still less satisfactorily. Recently, v. Dellinghausen (1933), further Bechhold and Ziegler 1906, v. Fürth and Bubanovic 1918, Stiles 1921, Stiles and Adair 1921, followed the changes of permeability by different methods, for instance, by measuring the electric conductivity of gelatine, imbibed with different equimolar salt solutions and afterwards fused into cylinders of equal length and cross-section. With chlorides of Li, Na and K one might expect the conductivity to rise in the series: Li < Na < K, corresponding to the electrolytic mobility of these cations. However, the contrary is true, i.e., the conductivity increases in the order: K < Na < Li, the order of increasing swelling or water intake, so that, according to the concept of Anselmino, the higher permeability would be brought about by enlarging the pores. Some observations of Friedman are also in agreement with this concept. They show that the diffusion rate of urea in gelatine can be increased about 33 per cent by the addition of saccharose, in agar about 49 per cent by glycerine, and 72 per cent by glucose. The effect is considered to be indicative that the non-electrolytes decrease the hydration of the solid phase, resulting in an increase in the size of the pores within the gel.

2. SOLVENT-LIKE MEMBRANES. The main starting point for experimental work on solvent-like membranes was the formulation of the lipoid theory by Overton, which states that the rate of penetration of water dissolved substances, chiefly of organic non-electrolytes into ani-

mal and plant cells, is due to their relative solubility in the so-called lipoids of the cell-surface. The presence in the superficial protoplasmic layer of such fat-like bodies can be concluded from a number of observations which will not be enumerated here.

a. *Permeability to non-electrolytes.* The relative solubility can be a factor influencing the rate of penetration in the following manner (Overton, 1895, 1899; Northrop, 1928; Collander and Bärlund, 1933; Irwin, 1928, 1931): a thin layer of water insoluble organic solvent may be put as a "membrane" between two aqueous solutions of the same substance, but of different concentration. Then, it may be reasonably assumed that a partition equilibrium at both interfaces between aqueous and non-aqueous solvent is rapidly attained. This equilibrium is characterized on both sides by having the same ratio of concentration in the two solvents (partition coefficient). Equilibrium having been established, diffusion through the organic solvent will go on because a concentration gradient has been set up, which, under many conditions, is proportionate to the partition coefficient. Thus, since diffusion usually proceeds slowly, it is the controlling factor in the whole process. But diffusion coefficients differ less from each other than partition coefficients do. On one hand, the molecular volume must have some effect on diffusion, since there is an inverse relationship between diffusion and molecular size; on the other hand, as has been mentioned already (see p. 58), even with great differences in the molecular weight diffusion rates do not differ much, by far not as much as, because of the variety of pore diameters, penetration across a porous membrane does. For this reason it seems questionable whether the correlation manifested in a number of physiological investigations between penetration power and molecular size can be interpreted at all as due to diffusion of substances across a layer of a second solvent. We must return to this problem later (see p. 95).

In passing, it should be recalled that there has been much question as to whether the lipoid theory should not be replaced by an adsorption theory in accounting for the entrance of dissolved substances into cells by a preliminary increase of their concentration at the interface between the aqueous medium and the protoplasm (I. Traube, 1904, 1908, 1909). Of course, it is outside the range of this paper to discuss this in detail. Nevertheless, since we are dealing here with the passage of dissolved substances across the phase boundary between two solvents, it must be remembered (see p. 62) that substances with apolar or approximately apolar shape like some hydrocarbons or their halogen derivatives (e.g., n-hexane, chloroform, carbontetrachloride, ethylene-chloride) become distributed through two solvents, like olive oil and water, without any adsorption at the interface (Harkins, 1926; Lazarew, Lawrow, and Matwejew, 1930).

Solvent-like "membranes" can be prepared from a very great number of different organic fluids; but if one wished to imitate physiological conditions it would be more reasonable to select as models substances resembling more or less in chemical and physico-chemical properties the plasma membrane constituents. When Overton created the term lipoid, or fat-like, he included those universal components of the stromata of the cells, namely, sterols and phosphatids. Such substances are capable of building up surface membranes (see p. 91). Yet there can be no doubt that the lipoids of cells represent a very complicated mixture of different substances, and although Overton and many of his followers were aware of this the lipoid theory of cell permeability has been tested mostly by comparing the permeation rate of organic nonelectrolytes with their relative solubility in olive oil or ether. As a matter of fact, the partition coefficients of these solvents, which unquestionably differ very much from the constituents of plasma membrane, often run parallel to a surprisingly high degree to the speeds of cell penetration, as may be followed, for instance, from the data of Collander and Bärlund (1933). (For a great number of partition coefficients see Henderson, 1930, and H. W. Smith, 1921.) However, Overton (1900), Ruhland (1908), Höber (1909) and many others have also found undeniably strong discrepancies between theory and observation. For example, in the field of vital staining, Overton discovered at an early date that the penetration of dyestuffs into cells is much more comparable to their entrance into a solution of cholesterol or lecithin in benzene or turpentine oil. Later, remarkable progress was made by Nirenstein (1920) who found that the different power of a great number of basic and acidic dyestuffs to stain Paramecia intravitally was closely comparable to their relative solubility in a mixture of oil, oleic acid and diamylamine. The presence of the oleic acid in this mixture was responsible for the uptake of basic dyes, the base diamylamine of acid dyes. We are justified in concluding from the efficiency of this model solvent that the molecule of the phosphatide contains both fatty acid and amine, both of which are split off partially within the substance of the plasma membrane. Obviously, these findings shift to some extent the fundamental principle of the solution theory of permeability, since real chemical affinities are substituted for less clearly defined affinities involved in the processes of solution. It is of particular interest that apparently the fatty acid and the amine do not neutralize each other in the mixture but are effective rather independently of each other.

Now, the ratio of affinity to either basic or acidic substances seems

to vary with different cells, so that the permeability to dyestuffs of one type of cell can be imitated only by a Nirenstein mixture with a higher content of either organic amine or fatty acid than that of another kind (Höber, 1930; Höber and Pupilli, 1931). Furthermore, it is not necessary that the substances utilized in permeation experiments be as decidedly basic or acidic as the dyestuffs for revealing comparable solution affinities in contact with some Nirenstein mixtures. It follows from experiments of Jacobs (1931), Wilbrandt (1931), Höber and Ørskow (1933), Collander and Bärlund (1933), Höfler (1933), that organic non-electrolytes, containing one or two amino groups in the molecule, differ in their permeation rate toward different kinds of cells, e.g., such as red blood cells of different species. It would seem that plasma membranes differ by corresponding amounts of acid, and, as a matter of fact, according to analyses of Collander and Bärlund, olive oil and olive oil plus 20 per cent oleic acid reveal a remarkably different solution power to substances like the amides of aliphatic acids or the alkyl derivatives of urea, as compared with alcohols with one or several alcoholic hydroxyl groups, monochlorhydrine, triethylcitrate and others.

In concluding this section concerned with solvent permeability to non-electrolytes, it would seem useful to recall briefly the oft-repeated discussion as to whether cellular phenomena can be pictured with greater success by the pore theory or by the solvent theory. This discussion arises chiefly from the fact that the type of experimental material determines largely the interpretation of the results. In the case of Beggiatoa, for instance, the results with the greater number of electrolytes tested favor the idea of a porous plasma membrane; on the other hand, Rhoeo or the skeletal muscle of the frog is penetrated by non-electrolytes at a high rate in accord with the relative solubility in lipoid-like solvent. From such findings the observers are induced to ascribe the seemingly anomalous behavior of the minority of substances either to the scarcity and inadequacy of knowledge about the porous structure and the molecular configuration and reactivity of dissolved substances, or to the great uncertainty about chemical and physico-chemical nature of the assumed lipoid solvent. Although it is true that our knowledge of these conditions is really very poor, it must be emphasized that, on one hand, lipoid-insoluble substances, which, in disagreement with the lipoid theory, enter living cells with remarkable speed, have a low molecular volume, and that, on the other hand, substances with a high molecular volume, which, in disagreement with the pore theory, enter easily, are revealed as highly lipoid-soluble.

From experiments of this kind it has been concluded for some time (Nathansohn, 1904) that neither the view of a solvent-like nor of a porous membrane would cover the facts, but that the main difficulties of both might be harmonized in the *mosaic theory;* this is the concept, that at least two kinds of structural elements exist side by side at the surface of the cells, which, as lipoid areas, can vary in their chemical and physico-chemical properties, and, as porous areas, can vary in size and shape of the pores, and both of which can occupy the surface with different percentage of the total area (Höber, 1926; Ruhland and Hoffmann, 1925; Mond, 1927; Bärlund, 1929; Jacobs, 1931; Schönfelder, 1930; Collander and Bärlund, 1933; Höber and Ørskov, 1933).

b. *Permeability to ions.* In general, organic liquids, which are immiscible with water, are poor solvents for strong inorganic electrolytes, since their low dielectric constant prevents any appreciable dissociation into free ions. This holds true mainly for the solvents of a chemically neutral character, like the neutral hydrocarbon oils, neutral esters, and others. The situation is different in the case of organic solvents having some acidic or basic properties, such as phenol, guaiacol, salicylaldehyde, toluidine, aniline, or solvents like the mixture of Nirenstein. These behave as weak electrolytes; they dissolve small amounts of strong electrolytes by chemical reaction. Through conductivity measurements it can be shown that the inorganic alkali salts, after shaking their aqueous solutions with solvents like guaiacol, benzaldehyde, p-cresol, have passed into these liquids in characteristically different amounts. We shall discuss later (p. 90) how far these findings are in favor of the lipoid theory in regard to the penetration of ions.

In general, organic liquids are quite suitable for taking up weak electrolytes, since undissociated molecules are very often soluble like other organic non-electrolytes. This is well known in the case of organic acids and bases, and it should be noted particularly that in many instances the speed of permeation of these compounds into plant or animal cells runs parallel with their respective relative solubilities in lipoid solvents. In dealing with an organic solvent, functioning as a membrane interposed between a solution of a weak acid or base and another inorganic solution, both at different pH's, the organic electrolyte is carried across the membrane and a very striking type of equilibrium, also encountered under physiological conditions, is set up. For instance, Osterhout (1925) and Osterhout and Dorkas (1928) have followed the partition of hydrogen sulfide between sea-water and the cell-sap of the marine alga, Valonia. When the sea-water is acid-

ified, so that the pH is identical with that of the sap (pH 5.8), H_2S will be in nearly the same concentration inside (95 per cent) as outside. If thereafter the outside reaction is changed by the addition of alkali to pH 8.5, the greater part of the undissociated molecules of the weak acid H_2S will dissociate in the sea-water, the equilibrium between inside and outside will be disturbed. Undissociated molecules will travel, from inside to outside, being reassociated inside and ionized outside until a new equilibrium has been established with H_2S inside only 4 per cent of that outside. At first sight this process would appear as if the weak acid had been shifted against its diffusion gradient, but this is not true. This experiment of Osterhout can be easily simulated by a non-living system; e.g., two volumes of the same aqueous solution of an indicator, each at different pH, one solution therefore being in the ionized, the other in the undissociated form, are separated from each other by a water-immiscible solvent, permeable to the indicator molecules (Irwin, 1927, 1928).

More complicated is the following shift of electrolytes across a solvent membrane (Teorell, 1933); carbontetrachloride as a membrane separates a solution of sodium salicylate and water, through the salicylate solution CO_2 is bubbled, which sets free salicylic acid, strongly soluble in the organic solvent and therefore able to pass into the water, where it splits into ions. Since the membrane is impermeable to sodium ions, permeable—at least indirectly—to hydrogen and salicylate ions, a Donnan equilibrium is established with a much higher concentration of hydrogen ions on the water side. The distribution of H ions depends on the particular pK of the organic acid, salicylic acid (pK = 3). In one of Teorell's experiments, after several hours of diffusion, the pH of the salicylate solution was about 6, that of water 3.2. Thus, the concentration of hydrogen ions was shifted about 1000-fold.

In some way comparable is a model system by which Osterhout and his associates (1933–34) have attempted to picture, at least partially, the conditions under which the special composition of the vacuole sap of the growing Valonia results. The sap differs from the surrounding sea-water mainly by its very high content of K and H and its low Na, while Cl has about the same concentration inside and outside (p. 80). In the model a mixture of guaiacol and p-cresol (G) is substituted for the protoplasmic layer, the alkaline sea-water by an aqueous solution of KOH and NaOH, the sap by water through which CO_2 is bubbled in order to imitate its production by the living cell. Now, the organic acid, guaiacol, combines with KOH to form potassium

guaiacolate (KG), which enters the organic solvent. Since the partition coefficient of KG between G and water is high, and KG only weakly dissociated in the organic solvent, its molecules move in the "membrane" along a fairly steep diffusion gradient to the other phase boundary, where, in contact with water plus CO_2 free guaiacolic acid and K^+ and HCO_3^- are formed (see Shedlowsky, 1934). Furthermore, the solubility of K^+ and HCO_3^- in G being small, the amount of potassium passing as KG in one direction predominates over the amount passing as K^+ in the other direction, so that it accumulates on one side. In contrast, the relative solubility of NaG is so far behind that of KG that the concentration ratio of K:Na in the "sap" becomes high. The volume of the sap increases with the rate at which CO_2 is bubbled through because the osmotic pressure is raised by the stream of gas. Thus, by intake of water the artificial cell "grows." As has been said before, although this model reproduces with remarkable fidelity certain features of cell function, the correlation is not complete; one point in particular is its inability to reproduce the intake of Cl^-.

With regard to sieve membranes, most evidence of ion permeability has been provided from the potential difference measurement. Similarly, potential differences develop at the two interface boundaries of an organic solvent separating two aqueous electrolyte solutions. Nernst (1892), Cremer (1906) and Haber (1908, 1909) have outlined and elaborated experimentally the theory that at the boundary of two phases, one being an electrolyte solution and the other a water-immiscible liquid solvent, a *phase boundary potential* is set up owing to the individual distribution tendencies of each kind of ion. It follows that in any liquid chain, consisting of a solvent membrane, interposed between two different electrolyte solutions, two different phase boundary potentials must be present, which are indicative of the different tendencies of cations and anions to pass the membrane.

An arrangement of such a biphasic battery system, most illustrative for the case in point, resembles the arrangement for studying the electromotive effect of concentration with dried collodion membranes (see p. 66); two aqueous solutions of the same electrolyte but at different concentrations are placed on either side of an intermediate layer of an organic fluid. As with solid membranes, the *"concentration potential"* (see p. 67) nearly approaches its thermodynamic maximum value. With salicylic aldehyde or guaiacol plus oleic acid as membrane and a concentration ratio of KCl 1:100 (Beutner, 1912, 1920, 1933; Loeb and Beutner, 1913) the chain develops about 100 millivolts, the

positive pole being on the low concentration side. This polarity holds also in the case of dried collodion and whenever the non-aqueous fluid acting as membrane has an acid character. If the acid be replaced by a basic one, e.g., by toluidine or aniline, the polarity is reversed as with collodion membranes impregnated with basic dyestuffs.

These observations may be advanced toward the elucidation of the underlying physico-chemical causes of the phenomenon. Beutner has proposed the following explanation: At the interphase between a KCl solution and salicylic aldehyde, K^+ enters the organic solvent at a higher rate than Cl^- because it can be exchanged for H^+, always present in the aldehyde owing to very small amounts of salicylic acid. In the concentration chain, c_1KCl | salicylic aldehyde | c_2KCl, in spite of the concentration difference between c_1 and c_2 at either phase boundary, an equal amount of K^+, dependent on the limited small contents of acid within the organic solvent, enters from both sides the same. Therefore, the chain functions according to the following scheme: c_1K in water | cK in salicylic aldehyde | c_2K in water, the potential difference being expressed by the formula: $E = 0.058 \log \frac{c}{c_1} - 0.058 \log \frac{c}{c_2} = 0.058 \log \frac{c_2}{c_1}$. It is evident that in this case the solvent membrane behaves like a *selectively cation-permeable membrane*. Correspondingly, basic solvents form *anion-permeable membranes*.

Potential differences can also be produced when a water-immiscible solvent is interposed between solutions of two different electrolytes (*chemical potentials*).

Comparing different sodium salts, the values of the chemical potentials follow each other in the well known order of the lyotropic series of anions: $SO_4 < Cl < NO_3, I < SCN$. Corresponding experiments with the chlorides of the different alkali metals gave the series: Cs < Rb < K < Na < Li. With regard to the aforementioned theory of phase boundary potentials, this signifies that in the series of anions and cations SCN^- and Cs^+, respectively, show the highest tendency to enter the organic solvent. As has been mentioned previously (see pp. 67 and 73), the reason for this is that in the series of anions and cations hydration decreases from SO_4 to SCN and from Li to Cs, respectively; therefore, SCN^- and Cs^+ have not only the highest adsorption affinity, but also the highest solubility in the hydrophobic organic solvents. With these properties is also bound up the marked tendency to escape the accompanying ion with opposite charge and lower solu-

tion tendency, even in the face of electrostatic forces. Accordingly, the very low conductivity of p-cresol is nearly doubled by shaking with a solution of NaCl, nearly tripled with an equimolar solution of KCl and the largest effect produced if an organic salt is used (Beutner). In addition to the above, if the influence of the anion and cation series upon the electromotive force of phase boundary is compared, it appears that in general the cations have the stronger effect in chains with acid solvents, the anions in chains with basic solvents. The influence with neutral solvents is small, for example, olive oil, but it is enhanced by the addition of oleic acid or of lecithin (Loeb and Beutner, 1913). Salts with one organic component usually reveal an especially strong effect either on the positive or the negative direction of the current, depending on whether the anion or the cation is organic.

Obviously, all these ionic reactions resemble the electromotive ones, which can be elicitated with more or less ease in the porous membranes (see p. 65).

Further, all these electrical phenomena, produced by the solvent membranes, resemble quite closely the conditions under which bioelectric potentials are generated or modified. An example to illustrate is the action of neutral alkali salts on the resting potential of the muscles, this action being localized in a supposed acid solvent similar to the aforementioned mixture of olive oil and oleic acid or lecithin present in the surface of the muscle fibres.

But, as has been pointed out by Beutner (1927, 1928), a layer of pure lecithin cannot exhibit the proper conditions of a solvent membrane. Chiefly from his studies on vital staining, Overton has already concluded that lecithin in contact with water in no wise displays dissolving properties like oil or oil plus lipoid because it becomes impregnated with water and gains in this way the dissolving properties of water. It is therefore understandable that a layer of pure lecithin, interposed between two aqueous solutions, behaves like some porous substance with water-filled pores. As a matter of fact, according to Beutner, a paste of starch as well as of protein, agar, kaolin or lecithin, separating, for instance, $\frac{m}{10}$ KCl and $\frac{m}{10}$ propylamine hydrochloride, gives rise to a potential of positive sign on the side of the organic salt, apparently because the diffusion velocity of K inside the porous channels in one direction exceeds that of the large sized and unadsorbable organic cation in the opposite direction. In contrast, in a similar system with lecithin in oil as intermediate layer, the positive pole is on the side of the KCl solution. These findings are of some physio-

logical interest, since studies of membrane potentials of living organs with relation to the influence of organic ions seem to favor the porous rather than the solvent-like membrane theory (Höber, 1926; Matsuo, 1923; Mond, 1924; Deutsch, 1925; Amberson and Klein, 1928).

Compound solvent-like membranes. As it has been shown previously (see p. 73), membrane potentials can also be developed with the same electrolyte solution on both sides, provided the membrane has an asymmetrical structure. This can be easily built up by placing a layer of a water-immiscible solvent of acid character adjacent to one of basic character. For instance, Beutner has measured the potential difference in a battery system like this: + NaCl | olive oil + phenol + oleic acid | olive oil + phenol + diamylamine | NaCl −, the negative pole being situated on the side of the alkaline solvent. This chain is comparable to a system containing an asymmetrical porous membrane: + NaCl | protein + NaOH | protein + acid | NaCl − (p. 74). Here the left side is cation-permeable, provided that the alkali is strong enough to shift the reaction to the alkaline side of the iso-electric point of the protein, while the right side is anion-permeable (see p. 74). Therefore, the left pole is positive, the right negative. Similarly, according to Beutner, the acid solvent in the first mentioned chain develops a stronger tendency to drive sodium ion into the aqueous solution than the alkaline solvent because there is a higher concentration of this ion.

3. Surface membranes. The artificial membranes hitherto described as representative models of the plasma membrane differ radically in spatial qualities from the natural objects inasmuch as the living cells are surrounded only by a very thin film, not to be compared with the bulk of substance contained even in a molecular sieve like the dried collodion membrane. The average thickness of such an artificial membrane has been found by Weech and Michaelis to be 9×10^{-3} cm., whereas Fricke (1926), from measurements of the electric capacity of a suspension of red corpuscles (assuming a value of 3 for the dielectric constant of the membrane) has calculated the thickness of the natural membrane to be 33×10^{-8} cm. = 33Å. This latter value places the thickness of the plasma membrane within the range of molecular dimensions, since a monomolecular film formed, for instance, by allowing palmitic acid to spread on an air-water interface, has a thickness of 24.2Å (Langmuir, 1926). However, from the very outset it has been stressed that a fairly complicated structure would have to be postulated for the plasma membrane, not only because substances like steroles,

phosphatides, proteins, presumably are involved, but also because chemical reactions in close association with enzymatic ones might take place therein or thereon. Nevertheless, it seems not amiss to refer in this review to the properties of monomolecular films, even though up to the present very little is known about their permeability and systematic investigations are lacking.

With regard to the foregoing chapters, naturally the first question to be raised is whether the permeability of surface films should be expected to resemble porous or homogeneous solvent membranes. Michaelis has given some lead toward an answer to this question: Even in a membrane consisting of a layer of liquid, functioning distinctly as a solvent medium, the penetrating molecules must pass through interstices between the molecules of the solvent as they have to do in diffusing across a molecular sieve like a dried collodion membrane. There is a marked difference, however, since the intermolecular spaces in a liquid membrane can easily be deformed and enlarged when penetrating solutes squeeze themselves between the movable molecules of the solvent, whereas the intermolecular spaces in the collodion membrane are preformed in the rigid structure of a solid phase. Considering all the evidence, favorable and unfavorable to the main theories of cell permeability, it is highly interesting to learn from numerous recent experiences that, while surface films under many conditions display distinct and fairly permanent structures, under other conditions they show properties of a system in vivid molecular movement as in a liquid. From this fact it appears that the properties of monomolecular films are particularly appropriate to the old concept of a solid-liquid state characterizing living substance.

According to Langmuir (1926) and Harkins (1926), a coherent monomolecular film of a normal saturated fatty acid can be obtained by allowing the acid dissolved in a volatile solvent such as benzene to spread on the surface of water. After evaporation of the solvent, a film remains in which the molecules are steeply oriented to the surface and tightly packed side by side either in perpendicular or slightly inclined position. Each molecule, because of its polar configuration, is constrained to keep one end, the COOH-group or the "head" in contact with the water surface because of its higher solubility, while the other end, the hydrocarbon chain or the "tail" is directed towards the air. The cross-section of one molecule of a normal fatty acid in the surface film has been measured and found to be 20.5 sq. Å $= 20.5 \times 10^{-16}$ (cm.)^2, the diameter of the cross-section about 5Å. If the hydrophilic COOH-group is replaced by the nitrile group, the area fixed to

the water surface increases to about 27.7 sq. Å because this hydropolar group has a larger size than the carboxyl group. Other active hydrophilic groups are: $-COOR$, $-COR$, $-CHO$, $-OH$, $-NH_2$, $-NCS$, $-NO_2$. The cross-sections vary over a wide range; for instance, in a condensed film of cholesterol the area has been determined to be 40.8, with lecithin 52 sq. Å.

Highly remarkable are the effects of temperature upon the molecular area (Adam, 1930, 1932; Rideal, 1926, 1932; Langmuir, 1933). For instance, in condensed films of calcium salts of fatty acids the film is distinctly solid below a special transition temperature, as may be detected by dusting the surface with talc and blowing softly; raising the temperature by about 2 degrees liquefies the film, and at the same time the molecular area increases, although the coherence of the molecules is retained. There is reason to believe that the solidity depends on adhesive or intermolecular van der Waals forces between tails and between heads of the molecules. With other films, such as palmitic acid, the area increases rapidly until the film is expanded to a "gaseous" film. Here the molecules may be thought of as lying flat with their whole length more or less parallel to the surface and with considerable space vacant between single molecules. From a number of different observations it must be concluded that during the transformation of the condensed film to the gaseous one the steep orientation of the molecules is gradually altered by thermal agitation, sufficient to overcome to some extent the lateral van der Waals forces, causing the chains to lie side by side, so that the flexible hydrocarbon tails begin to swing and rotate, whereby the distance between the chains is increased. In addition, the motion of the water molecules underlying the film will always have a disrupting action upon the adhesive forces between the chains.

There are still other factors tending to disperse the condensed structure of surface films. One of the most marked effects occurs upon addition of alkali to films of the higher fatty acids. Owing to the subsequent ionization of the carboxyl groups, similar electric charges on adjacent molecules are developed, and the lateral adhesion is diminished with increasing alkalinity. Similarly, with films of the amines the lateral adhesion is diminished by acid. No change is obtained with films of the alcohols upon addition of acid or alkali (Adam and Miller, 1933; Adam and Dyer, 1924). Whenever water-soluble end groupings of molecules are ionized under the influence of acid and alkali, molecular distances are enlarged, and spreading is increased.

Still another dispersing influence becomes evident where there is

adsorption of other surface active substances on the surface of water in contact with the close-packed molecules of the film. Adam and Miller, while studying the effect of pH on the coherent film of fatty acids, employed different buffer solutions. With phthalate buffers they found the unexpected result that molecular adhesion diminished as the solution became more acid. This expanding effect seemed to be due to the phthalic acid molecules becoming adsorbed at the surface, thus competing for the available space and causing the film molecules to adhere less strongly.

Experiences like these make it desirable to strive for a better understanding of the structure and behavior of films, composed by several kinds of molecules of varying size and activity, since, unquestionably, the phenomena of permeation into cells are concerned with films of considerable heterogeneity. In view of this the following experiments on the spreading of mixtures of some cellulose derivatives with castor oil (Adam, 1930–33) might have special interest. From the properties of monomolecular films of a number of cellulose derivatives it must be concluded that the cellulose chains lie flat in the water in a coherent film, with each hexose ring in contact with the water. When the molecules are pushed together by compressing the film laterally, effects are produced which can be attributed either to tighter packing of the chains or more inclined position of the rings due to unstable anchorage to the water. The main constituent of castor oil, triricinolein, is likewise supposed to lie nearly flat, so that lateral compression easily forces the chains into tilted positions. But upon the addition of the cellulose derivatives in considerable quantities, the molecules are squeezed into still smaller spaces on the surface, probably through the lateral adhesion between the chains of hexose groups compressing the entangled molecules of oil. In a similar way, intermolecular, interatomic and interionic forces of proteins, sterols, phosphatides and other constituents of the plasma membrane might act in modifying structure.

Now, up to the present the chief problem in this review, *the permeability of the monomolecular films*, has scarcely been touched; as a matter of fact we have to enter a rather unsettled state. However, the aforementioned observations have been quoted here because they might be helpful in throwing some light on the subject (for the following see Danielli, 1935 and unpublished).

From the first it has been emphasized that the structure of a surface film, like the film of a fatty acid, strongly resembles the *structure of*

a molecular sieve, as represented by the dried collodion membrane. Therefore, we may assume that such a structure is permeable only to those kinds of substances, the molecular sizes of which do not surpass a critical value. But, on the other hand, the permeability of such a film, as determined by the regular orientation of its molecules, would simply resemble that of a crystal with its regular crystal grating such as the chabasit previously mentioned (see p. 58). It could not be compared with the molecular sieve such as dried collodion or plasma membrane which allows a great number of molecules of different volumes to pass, owing to its variety of pore diameters (see p. 58). Here it might be helpful to recall the experiences suggesting the existence of thermal agitation of the hydrocarbon chains in a thin surface film, so that the adhesion forces are antagonized and the intermolecular distances are enlarged. For the magnitude of oscillation in such a layer of molecules is supposed to fluctuate around an average value, the more ample translatory movements being attained only rarely.

Now, the surface film, formed by a fatty acid, resembles not only in its structure the porous membrane, but also in its material the lipoid solvent-like membrane. Therefore, we must determine whether or not such a film as model of a plasma membrane could adequately explain the different *permeability to lipoid-soluble and lipoid-insoluble compounds*. Up to the present experiments dealing with rates of permeation of water-soluble substances across surface films with respect to their molecular volume are lacking. In one isolated instance Rideal (1925) and Langmuir (1927) have shown that the rate of evaporation of water from the surface covered by a film of lipoid material is appreciably lowered, while there appears to be no resistance offered to the passage of ether. From this we may conclude that such a lipoid film distinguishes between lipoid-soluble and lipoid-insoluble substances. The reason for this different behavior may be attributed to some sort of van der Waals forces, or, in other words, in an affinity of ether for hydrocarbon chains and a lack of such affinity in the case of water (Danielli). The same sort of affinity is presumably disclosed in the earlier described experiment of Adam and Miller, demonstrating the insertion of surface-active phthalic acid molecules into the layer of oriented fatty acid chains. In general, such intermolecular forces might be considered to be responsible for the varying penetration power of lipoid-soluble and lipoid-insoluble compounds.

The question arises whether *selective anion and cation permeability* might not be ascribed equally well to the properties of monomolecular

films. If we consider that the plasma membrane is very likely to contain as building material not only lipoids but also proteins, then it would be appropriate to derive the selective ion permeability from the properties of a protein film. The protein molecule is composed of a great number of fatty acid chains, linked together with different active bases or acidic side chains where ionization takes place, so that either positively or negatively charged groups could border upon the intermolecular and intramolecular spaces. According to the reaction inside the spaces, either positive or negative charges would prevail, so that either positive or negative ions would be prevented from entering. An iso-electric behavior could even be manifested. Because of electrolytic dissociation, water dipoles could be fixed with a resulting layer of water filling the porous structure. Therefore, such a film must reveal properties similar to those of a dried collodion membrane; it must be permeable to water; it must be the seat of a potential, and depending on the pH it must permit passage to either cations or anions.

Another problem in this field, which has been successfully approached, is that of *the different temperature coefficients of penetration*. A high coefficient is usually attributed to a chemical reaction connected with the permeation process. According to Danielli and Davson, high temperature coefficients may be deduced also from properties of the film without the necessity of introducing chemical reactions. Suppose that only molecules having energy of translation greater than a critical value are able to pass the film; this critical value will be the smaller the less resistance the molecule meets in penetrating the film. Therefore raising the temperature must favor the passage of a greater fraction of the total number of molecules in the case, where the critical energy is large (where the permeability is small) than in the opposite case. This deduction is in agreement with several physiological observations. For example, low temperature coefficients are found when water enters erythrocytes which are highly permeable to it, and the reverse when water enters *Arbacia* eggs which have a relatively small permeability to it. Jacobs has also noted, in comparing the penetration of different solutes into erythrocytes, that those which enter with the greatest difficulty have the highest temperature coefficients.

Finally, if we wish to harmonize porosity with that of solvent-like behavior in the plasma membrane, we will find that a film composed of a *mosaic of molecular aggregates of protein and of lipoid material* offers an adequate concept (Brooks, 1934).

Nearly all the features of permeation through monomolecular films have been inferred but not demonstrated experimentally, largely because, until now, the studies have been confined almost exclusively to films produced at the interface between water and air instead of between two solvents. In physiology only the latter are important since the plasma membrane is always an interfacial membrane between two aqueous solutions. But formation of interfacial films between water and water is fraught with technical difficulties. Obviously, the monomolecular films of fatty acids can exist only because their molecules are oriented and fairly fixed in position by interfacial forces, the heads following their hydrophilic, the tails their hydrophobic character. The problem might be solved by the formation of a bimolecular film, the two layers of heads bordering two aqueous solutions, the two layers of tails touching each other. Blodgett and Langmuir have devised such an arrangement. In addition, Danielli and Harvey (1935) have worked out some conditions for constructing an interfacial film which portrays physiological properties more faithfully than interfacial films of oil or hydrocarbon. They have observed that at the interface between a surface inactive hydrocarbon, like brom-benzene and water, the tension is of the order of 39 dynes. Compared with this, the tension between a cell-fat, like the oil in a fish egg, is only about 9 dynes. By various means Harvey and his associates have shown that the tension at the surface of a number of cells is still smaller, i.e., less than 2 dynes, while, according to Danielli and Harvey (1935), the tension between the oil and the aqueous contents of the fish egg is even less than 1 dyne. This is due to the presence of a highly surface active globulin-like protein in the aqueous egg contents. They have demonstrated that this globulin is adsorbed at the oil-water interface. When there is a high surface tension as between water and a hydrocarbon like brom-benzene, a sheet of globulin adsorbed at the interface undergoes rapid denaturation and coagulation; on the contrary, normal solubility and surface activity of globulins are preserved at the lower tension between egg oil and water. This condition is even more or less preserved after the addition of egg oil, lecithin or cholesterol to hydrocarbon. Danielli and Harvey therefore conclude that the surface of the cells is built up of a layer of an oil, which, by virtue of its relatively low surface tension gives fairly coherent film. This layer could be even more stable if it were covered with a layer of protein which would lower the surface tension still more.

CONCLUSION

The foregoing article presents far more physical chemistry than physiology of membranes. This method of approach has been purposely adopted in order to focus attention on the very stimulating studies on artificial membranes as models for the natural, and to point out fruitful lines of future research. Unfortunately, the field of physical chemistry contains numerous gaps because physiologists in following their special problems have usually not been inclined to dive deeply and thoroughly into the subject, but have confined themselves to a fairly superficial study. This is especially evident in reviewing the extensive literature concerning lipoid-solubility in relation to cell permeability. The idea has been advanced, that only a complicated mixture of different organic solvents whose solvent power approaches more or less a real chemical affinity can be compared with the lipoids of the plasma membrane. Further, comparing the plasma membrane with a molecular sieve, e.g., with the dried collodion membrane, we cannot be satisfied in visualizing the membrane as a rigid layer pierced by channels, but we must know the shape, the nature of its electric charge, the polar and non-polar adsorption affinities of the walls, the intimate structure of the double layer and how to produce reversible changes of the pore diameters. Moreover, we shall face quite an unsettled field when we try to imitate plasma membranes with the interfacial films, perhaps more comparable than any other models because of their thinness, reactivity and properties mediating between the concepts of pore and solvent permeability. But, even after all the refinements in model membranes designed to make them as nearly like their natural counterpart as possible, they must still be regarded by physiologists as rather poor and inflexible imitations. This is most forcibly emphasized when one considers, for example, the extremely specialized permeability of red blood cells in different species. To explain this would require the postulation of special chemical or enzymatic activities resident in the interfacial membrane. Still more noteworthy is that permeation often signifies the transport of solutes dependent upon suitable energy supply by metabolic reactions. Evidently these and similar facts cannot be covered by the properties of any artificial membrane.

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FETAL CIRCULATION AND RESPIRATION

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Not only have different mammals different periods of gestation but they are born in very unequal degrees of development. These facts have constantly to be borne in mind when observations made upon the physiology of the embryo in one form are compared with those made in another form. To take an example: the sheep, though it has a longer period of gestation than the dog and a shorter one than man, is born in a much more developed condition than either.

I am indebted to my colleague, Prof. H. A. Harris, for the information on which the following calculation is made. If the ossification of the distal end of the femur be taken as a standard, this takes place 30 days after insemination in the rat, 46 days in the guinea pig, 74 days in the dog and cat and 98 days in the sheep and goat. Thus one day of fetal life in the rat is developmentally equivalent to about 1.5 days in the guinea pig, 2.5 days in the dog and cat and 3.3 days in the sheep and goat.

It seems best to work out the physiology of one species as completely as possible and having done so to make comparisons with others, using it as a standard and always recollecting the difficulties introduced into such comparisons contingent upon the considerations to which attention has been drawn.

Circumstances have to some extent dictated the form chosen in many of the experiments to which allusion will be made; in most of them either the sheep or the goat has been used. These have the advantage of being large enough for the application of many techniques which are unsuitable in the case of small animals, and for the systematic study of the progress of the development of physiological processes it is desirable to use an animal with a longish period of gestation. In the sheep and the goat this period is about 150 days. As against this, the sheep and the goat have certain disadvantages, not shared by, say, the pig, but on the whole Cohnstein and Zuntz (1884) were not ill-advised to choose the sheep as their subject when they made their classical attempt to study fetal respiration.

The present article will in the main take the form of putting before the reader such few facts as are known about the circulation and respiration in the sheep and goat, with notes on other animals by way of comparison.

The circulatory and respiratory functions are even more closely interwoven in the fetus than in the post-natal animal; this being so, no apology will be made for regarding the two as inseparable.

Of the basic data with regard to the fetal circulation and respiration perhaps the first to be considered is the fetal blood volume.

TABLE 1

SERIAL NUMBER OF GOAT	AGE OF FETUS	BLOOD VOLUME	VOLUME OF CIRCULATING BLOOD CC. × 100 DIVIDED BY THE WEIGHT IN GRAMS OF	
			Fetus	Fetus + placenta
	days	*cc.*		
17	68	16.3	21.8	8.0
6	71	40.4	40.4	8.8
7	77	39.9	23.9	8.6
8	85	64	23	8.6
10	101	112	17.3	9.5
12	113	145	11.6	6.8
14	126	204	14.2	9.9
21†	136	136	14.2	9.6
22	144	428	14.7	11.95
30*	148 (1)	563	16.7	
29*	149 (2)	392	11.2	
24*†	151 (1)	161	9	
20*	156 (2)	720	15	

* Nos. 30, 29, 24 and 20 are kids. The figures in brackets in column 2 are the ages in days after birth.

† Triplet.

The fetal blood volume has been measured by Elliott, Hall and Huggett (1934) in goats during the progress of fetal life. The method used was that of Keith, Rowntree and Geraghty (1915), but the dye in this case was not Vital Red but chlorazol sky blue FF.

Table 1 gives the blood volume from week to week.

It is usual to express the blood volume either as a percentage of the weight of tissue through which the blood circulates, or as a function of the surface of the body. Both these conventions in the case of the fetus present difficulties. The embryo has no surface, if the word surface be used in the physiological sense, intended to convey to the

reader the idea of an area from which heat is dissipated. There seems little point therefore in calculating the relation of the blood volume to the cutaneous area. As regards the blood volume considered in relation to the body weight, two possibilities exist. The body weight in the case of the fetus is not the same thing as the weight of material through which circulation takes place, unless indeed the placenta be regarded as an integral part of the fetus. We may therefore calculate the fetal blood volume as a percentage of the weight of the actual embryo or as a percentage of that of the embryo plus the placenta. In either case the calculation is of some interest, but perhaps more interesting is the comparison of the two.

The youngest embryos in which the blood volume was measured were about ten weeks old. In all cases the blood volume in cubic centimeters was over 20 per cent of the weight of the fetus in grams, and indeed that was true up to the twelfth week. Doubtless the explanation of this apparently large blood volume lies in the fact that the placental mechanism, of which the blood stream forms a part, is on the whole developed before the fetus, and one might expect at this stage to find a great proportion of blood in the placenta. The blood volume expressed as a percentage of the fetal weight gradually decreases till at birth it is about 14 to 15 per cent.

Considered as a percentage of the weight of the fetus plus that of the placenta, the blood volume is much more uniform, being through most of fetal life about 9 per cent with a tendency to rise gradually.

If taken at their face value, the above data suggest that at any time a considerable quantity of blood may be in the placenta and that, relatively to the quantity in the fetus, this decreases as pregnancy proceeds. We have but few data on this subject. During the past winter the present author set out to acquire some data upon sheep, but he must confess to not finding himself quite happy with the chlorazol sky blue. A single determination with the "washing out method" gave rather lower results. It must be remembered that the errors in the dye-injection method would naturally be such as would yield too high results and those in the washing out method such as would give too low an answer, therefore provisionally it may be taken that the correct figure is between the two. Moreover, although the differences between the results given by the two methods seem considerable, the difference is less important than the similarity. The striking point is that a quarter of the blood or more is in the placenta (including the vessels of the cord) and is therefore outside the fetus itself (table 2).

The minute volume. Granted that the fetal blood volume in cubic centimeters is about 9 per cent of the combined fetal and placental weights in grams, the question naturally arises: At what rate is this blood driven round the body? An attempt has been made to answer

TABLE 2

SERIAL NUMBER OF SHEEP	FETAL AGE	METHOD	PERCENTAGE OF BLOOD IN	
			Body of fetus	Placenta + umbilical vessels
	days			
6	84	Dye-injection	51	49
7	96	Dye-injection	48	52
11	111	Dye-injection	66	34
19	137	"Washing out"	73	27

TABLE 3

	SERIAL NUMBER	FETAL AGE	AGE FROM BIRTH	OUTPUT PER MINUTE	OUTPUT IN CC.	
					Per gm. body weight	Per gm. of fetus + placenta
		days	*days*	*cc.*		
Fetus	18	89		26	0.13	0.06
	19	103		70	0.13	0.07
	32	115		217	0.18	0.16
	21	136		154	0.18	0.17
	22	144		262	0.12	0.11
	36	150		257	0.18	0.17
Kid	27		<1	302	0.14	
	33		1	596	0.20	
	28		3	322	0.19	
	34		6	570	0.15	
	31		15	773	0.13	
Goat	Mean of 21 extremes		Adults		0.27 0.11–0.44	

this question, the animals used being goats (Barcroft, Flexner and McClurkin, 1934). The method used was plethysmographic, a method which did not promise too well. Calibrated on animals of various sizes, however, against Fick's method, it gave results which were about 15 per cent too low. That the cardiometer should give results which

were too low was to be expected; it only professes to measure, even if it records correctly, the stroke volume minus the volume entering the auricle during the stroke and minus the coronary flow. But there are other dangers: 1, that the cardiometer may restrict the flow, and 2, that owing to the technical errors of the instrument it may give a wrong measurement of the amount of blood which is actually passing. It was somewhat surprising to the authors that the values obtained with the cardiometer agreed so nearly with observations by Fick's method. Actually the observations recorded in table 3 are measurements of the output made by the cardiometer, increased by 15 per cent.

Calculated as a function of the body weight of the fetus the figure is of about the same order from the 12th week onwards, tending to rise somewhat as time goes on, but calculated as a function of the whole tissue through which the blood circulates the answer is otherwise.

TABLE 4

SERIAL NUMBER OF GOAT	FETAL AGE	PROBABLE BLOOD VOLUME	ACTUAL OUTPUT	PERCENTAGE OF BLOOD VOLUME CIRCULATING PER MINUTE
	days	*cc.*	*cc.*	
18	89	47	26	55
19	103	88	70	80
32	115	163	217	133
21	136	101	154	152
22	144	254	262	103
36	150	157	257	163

Relative to the weight of tissue through which the blood circulates, the cardiac output is an ever increasing quantity during fetal life. At the end of fetal life about three times as much blood is irrigated through each gram of tissue as at the 89th day. Remembering that the blood volume is approximately 9 per cent of the gross weight of fetus + placenta, it is possible to calculate roughly the proportion of the total blood which is driven round the system per minute at various ages. The result shows as smooth an increase as can be expected considering the data from which the calculation was made, and gives the general result that about half the blood makes the circuit in each minute in the 89th day, whilst at birth the quantity circulated is one and a half times the blood volume (table 4).

In considering these calculations it must not be forgotten that the output of the heart is that of both ventricles. In using the term

minute volume in man, the expression is applied to the output of only one ventricle, which at rest is not very different from the blood volume. In man the total output of both ventricles at rest for a person whose blood volume is 4 litres would be in the vicinity of 8 litres per minute. The output would therefore be greater relatively to the blood volume than in the heart of the fetal goat at birth.

The arterial pressure. The speeding up to the circulation is in harmony with the progressive rise in arterial pressure which takes place as the embryo grows in age.

A number of authors have worked upon fetal arterial pressure from various points of view and their results will be considered in connection with the problems at which they worked. The results are summarized by Schlossmann (1932) and vary from 83 mm. as observed by Cohnstein and Zuntz in the sheep to 10 mm. found by Corey for the rat. The ages of the fetuses are not given. Schlossmann observed an arterial pressure of 50 to 60 mm. in fetal goats near term, while the fetal

TABLE 5

Arterial pressure in sheep embryos

Fetal age in days	49	101	120	123	137	138	140
Arterial pressure in mm. Hg	20	34	46	50	72	68	76

sheep of 146 days had an arterial pressure of 55 to 60 mm. as compared with 80 mm. at 151 days. But the literature lacks information about the relation of the establishment of arterial pressure to fetal age. In table 5 the observations are all those of carotid pressure taken with the mercurial manometer except that at 49 days, which was taken by the insertion into the umbilical artery of a hypodermic needle, fitted to a micro-manometer of saline solution.

G. A. Clark (1932, 1934) has worked upon the fetal arterial pressure in cats and dogs toward the end of pregnancy. The precise fetal ages are not stated, but the blood pressures are definitely lower than those given above for the end of the gestation period in the sheep. They range about 40 mm. Hg for the dog and 30 mm. Hg for the cat. In Clark's animals the anesthetic given to the mother was chloralose, but the difference in blood pressure is probably attributable less to difference of technique than to difference in fetal development at birth. A pressure of 34 mm. was given in the sheep at 101 days and the pup a few days before birth is probably no more developed than the sheep

at a fetal age of 101 days. The 30 mm. observed in the cat near to birth is attained in the sheep at about 90 days.

Venous pressure. "Cohnstein and Zuntz," in the words of Clark (1932), "made the very interesting observation that the venous pressure in the fetus is much higher than in the adult and approximates to a value one-half of that of the fetal arterial pressure." This observation was of course made in sheep and Cohnstein and Zuntz record a venous pressure of 30 mm. Hg. Our own observation made on a single occasion gave a pressure in the umbilical vein of 20 mm.

The "work" done by the heart. The increase in the work done by the heart as the fetus grows may be calculated according to the classical method of multiplying the weight of blood put out per minute by the pressure against which it is expelled. There are no separate measurements of the pressure in the right and left ventricles respectively, but as the two discharge into a common vessel, the aorta, though by different paths, the pressure cannot be very different in the two compartments. This assumption is borne out by the fact that there is no disparity in the thickness of the walls of the left and right ventricles respectively during fetal life. If then the heart of goat 18 at 89 days be compared with those of goats 22 and 36, at 144 and 150 days respectively, there is seen to be a tenfold increase in the output and a rise of pressure from about 30 mm. to about 70 mm. The proportionate increase in work would be of the order of 30:700—more than twentyfold.

The course of the stream. A good account of the theories with regard to the "crossing of the streams" of blood coming from the inferior vena cava and the head respectively in the right auricle, is given by Pohlman (1907) together with the literature on the subject up to 1907.

Pohlman himself observed the distribution of starch grains injected into the venous system and concluded that the blood from the various venous sources mixed completely in the right auricle and that the head did not receive blood of a better quality than that which passed down the abdominal aorta.

In some animals,[1] e.g., the guinea pig, the most casual observation of the fetal heart in action shows that the two sides differ in colour, the left side being bright and the right dark. This seems to favour some crossing of the streams in the right auricle. The classical description is that the blood from the inferior vena cava goes through the *foramen*

[1] I am indebted to Dr. B. M. Patten for pointing out to me that anatomically the hearts of the guinea pig and sheep lend themselves more to the classical theory than does that of man.

ovale to the left auricle thence to the left ventricle and so up the aorta, whilst the remaining blood reaches the right ventricle, goes up the pulmonary artery, thence along the *ductus arteriosus* and for the most part also finds its way into the aorta.

We may speak then of an "upper" and a "lower" circulation. The upper circulation consists of that blood which leaves the left ventricle and finds its way along those arteries which spring from that part of the aorta proximal to the entry of the *ductus arteriosus*. These are the carotids and the brachials and the coronary artery, and they supply the head, the arms, the heart and a great part of the upper portion of the trunk, through mammary (internal and external) arteries. The extent to which the somatic muscles of the trunk form part of the "upper circulation" is shown also by the great size and obvious importance of the azygos vein and its confluent vessels representing as they do the cardinal sinus system. To me the importance of all this part of the "upper circulation" was a little surprising. Coloured gelatine injected from the left ventricle with the aorta ligatured just above the entry of the *ductus arteriosus* goes not only to the brain, but also to the cord as far down as about the middle of the thoracic region.

"The lower circulation" consists principally of the placenta but in addition it includes the lower limbs, the lower part of the body, the abdominal viscera and the lungs. The liver stands in a peculiar position. In one sense it is part of the lower circulation, but almost all the blood which traverses it has already traversed some other organ since leaving the heart, either the intestine, etc., or the placenta. We do not know just how much comes from each source, but probably the greater part comes from the placenta. In the ungulates which are described as having no *ductus venosus*, and in which therefore all the placental blood irrigates the liver, the placental blood must form the overwhelming proportion of the whole supply to that organ.

If the separation of the upper from the lower circulation was complete, as it would be if *a*, the aorta was ligatured just proximally to the entry of the *ductus arteriosus*, and, *b*, the crossing of the streams in the right auricle was absolute, then every corpuscle would take a figure of eight course round the body, going in succession round the upper and the lower circulations. At each circuit through the lower circulation a portion of the blood would go through the placenta. Incidentally of course the volume of blood leaving the heart for the "upper circulation" in a given time would be equal to that reaching the heart from the same, and also equal to that leaving the heart for, and reaching the heart from the lower circulation.

The interesting question is how far is the picture realized? What proportion of the corpuscles do actually make the figure of eight circuit?

At present we are not able to give a completely satisfactory answer to the question. Probably there is no hard and fast answer. On general grounds, the crossing of the streams depends upon the shape of the heart, the positions of the orifices of the *foramen ovale* and the bicuspid valves relatively to those of the great veins. It is unlikely that these orifices remain constant in their relative positions. Movements of the heart, owing to changes in the position of the fetus, and altering effects of gravity according to the position of the mother may naturally be expected considerably to affect such a mechanism, if indeed the crossing of the streams can be dignified by that word. Yet some sort of attempt may be made to obtain light on the subject, and having been made it does not fail to produce some illumination. The method of approach is to choose certain salient points in the system where two streams meet, and analyze the blood in each stream and in the confluent for some suitable substance, oxygen being in this case the one which has been used. The principle scarcely needs labouring; if the content, say of lime, was very different and was known in each of two confluent rivers and also in the stream formed by their union, it would be a simple matter to tell the relative volumes of water running in the two streams.

It is not possible always to attain even to this ideal method of approach, but let us put together some of the scraps of knowledge available. In the first place can it be shown quantitatively that the blood which goes to the "upper circulation" really does contain more oxygen than that going to the "lower circulation?" As the result of six experiments, Huggett (1927) found that the blood from the carotid contained 5.9 volumes per cent of oxygen and that from the umbilical artery 2.9. The blood from the umbilical vein contained 8 volumes per cent.

This picture is quite different from that given by H. B. Kellogg (1930) who, in fetal dogs, found no difference between the blood of the right and left ventricle. In both cases the blood was almost completely reduced, containing on the average only 2 to 4 volumes percent—probably therefore about 12 per cent saturated. The oxygen capacity was not given.

Table 6 gives some recent analyses.

Our answer obviously is that there is on the whole a difference—and therefore there is some amount of crossing of the streams. Is this crossing complete? If it were, clearly the carotid blood would have the same composition as the blood from the thoracic portion of the inferior vena cava, whilst the blood in the abdominal aorta would be

identical with that coming from the jugular and azygos veins. The first comparison is the easier and may be taken first. Is the blood in the carotid the same as or different from that in the thoracic portion of the inferior vena cava?

The thoracic vena cava blood is on the whole better aerated than that of the carotid, though only in one case was the difference very great and in another it was non-existent (table 7).

TABLE 6*

SERIAL NUMBER OF SHEEP	FETAL AGE	PERCENTAGE SATURATION OF BLOOD WITH OXYGEN		
		Carotid	Aorta (umbilical artery)	Difference
	days			
11	111	72	48	24
14	112	64	64	0
16	124	41	23	18
19	137	22	10	12
27	138	50	43	7

* The data in tables 6, 7, 8, 9, 14 and 15 were obtained in collaboration with G. S. Adair, E. H. Hale, K. Hayashi, L. Lequime, A. V. O'Brien, W. S. Root, from a research as yet unpublished.

TABLE 7

SERIAL NUMBER OF SHEEP	FETAL AGE	PERCENTAGE SATURATION OF BLOOD WITH OXYGEN		
		Thoracic inferior vena cava	Carotid	Difference
	days			
11	111	70	72	−2
14	112	73	64	9
16	124	56	41	15
19	137	27	22	5

Let us turn now to compare the most arterial blood which reaches the heart with that which feeds the "lower circulation" (table 8).

On the whole therefore the blood reaching the heart from the lower circulation contains more oxygen than that leaving it either for the upper or lower circulation, but the disparity is less between it and the carotid blood than between it and the blood in the abdominal aorta. In the four experiments in which we have data for both, the disparity in the first case ranges from − 2 to 15 per cent, in the second from 9 to 33 per cent.

The comparison between the blood in the jugular vein and that in the abdominal aorta rather breaks down because the former sometimes contains more oxygen than the latter, and we are left to suppose that the jugular vein does not provide a fair sample of the blood reaching the heart from the upper circulation; the differences between the blood in the jugular and the azygos veins which of course should come into the picture have not yet been studied. The figures, such as they are, are given in table 9.

TABLE 8

SERIAL NUMBER OF SHEEP	FETAL AGE	PERCENTAGE SATURATION OF BLOOD WITH OXYGEN		
		Thoracic inferior vena cava	Aorta (umbilical artery)	Difference
	days			
11	111	70	48	22
14	112	73	64	9
16	124	56	23	33
19	137	27	10	17
29	138	48	18	30

TABLE 9

SERIAL NUMBER OF SHEEP	FETAL AGE	PERCENTAGE SATURATION OF BLOOD WITH OXYGEN	
		Jugular vein	Aorta (umbilical artery)
	days		
11	111	39	48
16	124	33	23
19	137	16	10
29	139	41	18
Goat 32	115	32	19

Perhaps the most surprising feature in the above data is the low oxygen content often shown by the blood in the abdominal aorta. Of course the corpuscles have for the most part suffered two drops in oxygen level since leaving the placenta; first, they have lost oxygen through mixing with the blood from the abdominal vena cava and secondly, they have most of them made the circuit of the upper circulation. Regarding the placental circulation as the preponderant feature in the lower circulation, of course it is all to the good that the oxygen in the abdominal aorta should be as low as possible, but little oxygen is left for the organs dependent on the abdominal aorta for their oxygen supply.

Taking the above and other facts into consideration, it seems that provisionally we shall not be far wrong if we portion out the blood in more or less the following way. Taking the blood flow per minute round the "upper" and "lower" circulations as being not very unequal, we may picture that out of six cubic centimeters coming to the heart three come from each. Of the three which come from the lower circulation, two go to the upper and the third goes back to the lower, and likewise of the three which come from the upper, two go to the lower and the third returns to the upper.

Therefore to take an example: in such an experiment as goat 36 (table 4) where the quantity of blood which traverses the heart is about 258 cc. per minute (257 actually), 129 cc. would circulate through each circulation and of the 129 cc. which came from the "upper circulation," 86 would pass on the "lower circulation," with 43 cc. of blood from the inferior vena cava. It would find its way into the right ventricle and be driven up the pulmonary artery and for the most part along the *ductus arteriosus*. The question naturally arises, is the *ductus arteriosus* large enough at the end of pregnancy to serve for the passage of so much blood?

In a single experiment some observations were made to test this question by perfusing blood through it. The pressure at which 120 cc. per minute traversed the *ductus arteriosus* was, for the dead vessel perfused with cold blood, 82 mm. Hg but it must be borne in mind that the pressure on the distal side was atmospheric; with 103 mm. perfusion pressure and 69 on the distal side, only about 80 cc. of blood per minute went through the vessel.

Taking the blood which traverses the lower circulation in a late sheep's or goat's embryo at 120 cc. per minute, the question arises: What proportion of this goes through the placenta and what proportion is used for supplying the lower part of the body with oxygen? The uncertainty in answering this question depends upon the difficulty in obtaining fair samples of blood from the abdominal vena cava. In theory it should be easy to get samples from the thoracic inferior vena cava, the umbilical vein and the abdominal vena cava and to determine the proportion in which the last two must mix to make the first. Actually, the comparison has not been made with any accuracy. This however can be said. The blood in the abdominal vena cava cannot contain more oxygen than that in the abdominal aorta—which as we have seen is strikingly little—and likewise it cannot contain less than nothing. We have therefore a superior and inferior limit for the oxygen

in the abdominal vena cava. The results would work out somewhat as given in table 10.

Evidently the greater portion of the blood which flows down the abdominal aorta goes to the placenta. The disparity between the figure for the superior and inferior limits of the ratios of placental blood to body blood, as given in the above table, may suggest a greater difference than actually exists. Thus to take experiments 14 and 19, the data mean that of 100 cc. of blood going down the abdominal aorta not less than 69 cc. go to the placenta and not more than 80 cc., and even in sheep 11, the disparity, which on the ratios looks so large, is actually as between 71 and 89 per cent of the aortic blood going to the placenta, whilst in sheep 16 the placental flow would have been between 55 and 64 per cent of the whole.

If we say that two-thirds of the blood which passes down the aorta irrigates the placenta we shall not be exaggerating its function.

TABLE 10

SERIAL NUMBER OF SHEEP	FETAL AGE	RATIO OF BLOOD FLOW THROUGH PLACENTA TO THAT THROUGH REST OF LOWER CIRCULATION	
		More than	Less than
	days		
11	111	2.4:1	8:1
14	112	2.2:1	4:1
16	124	1.1:1	1.8:1
19	137	2.2:1	4:1

We are clearly not in a position to make any statement as to the variation of this ratio with fetal age.

Looked at from a distance, two points stand out: first, how completely the lower circulation is, physiologically speaking, a "pulmonary" circulation, and secondly, the meanness, both in quantity and quality of the blood supplies, of the circulation to the lower part of the body.

Metabolic rate. We are, however, in a position to say something about the metabolic rate, at all events to discuss the order of quantity involved.

If we take the blood flow through the heart as 0.15 cc. per gram of fetus per minute, from the calculations made above, the half of that would go to the "lower circulation" of which two-thirds would go to the placenta. The placental flow would therefore be two-sixths of the whole or 0.05 cc. per gram of fetus per minute. On this basis we should obtain the values for the metabolic rate given in table 11.

Apart from one divergent observation the results are remarkably uniform, considering the ruggedness of the data from which they were obtained, giving a result of 0.0026 cc. ±0.0006 per gram per minute. In the paper by Barcroft, Flexner and McClurkin, the metabolic rate for goats is calculated making different assumptions from those made above and is found to give essentially the same answer.

The important point with regard to the value of 0.0026 cc. for the metabolic rate is its lowness. The average value for five kids, under anesthetic, whose ages varied from 20 hours to 15 days was 0.08 cc., whilst for 21 adult goats, not under anesthetic nor even under basal conditions, but just at rest standing or lying, was 0.085 cc. per gram per minute.

TABLE 11

SERIAL NUMBER OF SHEEP	FETAL AGE	WEIGHT	OXYGEN IN CC. PER 1 CC. BLOOD			OXYGEN USED PER GRAM OF FETUS PER MINUTE
			Umbilical vein	Umbilical artery	Difference	
	days	*kgm.*				
2	58	0.05	0.078	0.028	0.05	0.0025
8	98	0.79	0.088	0.048	0.04	0.0020
11	111	1.4	0.113	0.069	0.044	0.0022
14	112	1.5	0.157	0.104	0.053	0.0027
16	124	1.65	0.174	0.052	0.122	0.0061
19	137	3.6	0.068	0.017	0.051	0.0026
27	138	3.6	0.105	0.059	0.046	0.0023
29	144	4.5	0.102	0.038	0.064	0.0032

The figures given in the literature for the metabolic rates of sheep vary greatly. Reiset, quoted by Schäfer (1898) gives 0.0057 cc. per gram per minute, whilst the figure given in Smith's *Veterinary Physiology* (1921) works out to 0.028 cc.

For the low figures obtained for the fetus there appears to be a reason; the fetus has no cooling surface and is under no necessity to keep up its own body temperature. The heat which it produces must ultimately be dissipated by the mother and the greater the heat production in the fetus the greater the burden thrown upon the parent. That the metabolism of the fetus is much reduced is evident from its flaccid condition, and a particularly beautiful demonstration of this fact was furnished by my colleague, Dr. D. N. Barron, who, applying the "amplification technique" observed the electric waves generated in the muscles of the fetus while normally attached to the cord. The fetus was alternately

lifted out of the bath of warm saline (in which the sheep was placed) and lowered into the same. In the warm bath no evidence of muscular tone was found, but as soon as the fetus was taken out, marked evidence of tone appeared in the muscles. The alternation was carried out many times.

The pulse rate. The following values for pulse rates for man, the ox and the dog have been collected by Clark (1927) as representative figures; to these we have added some for the goat and rat.

	PULSE RATE		
	Fetus	New born	Adult
Man	135*	112	70
Ox	161	141	50
Dog	120–170	160	100
Goat	120–246	145–240	
Rat	96–256		184–264
Monkey	100–200		140–240

* Five to nine months.

Though there are considerable variations in the rate of heart beat during fetal life, the pulse rate is of much the same order in the fetuses of most animals so far investigated and has no relation to the ultimate pulse rate in the adult. This may be faster than the fetal pulse rate as in the case of the rat and birds or much slower as in the case of man and the ox. In the monkey the fetal pulse rate is much slower than that of the mother until just before birth.

The variations of the pulse rate with the fetal age have been studied in the rat by Corey (1932) who observed certain periods at which the pulse rate was maximal corresponding roughly to crown-rump lengths of 16, 23 and 34 mm.

The striking point was that these periods corresponded to simultaneous maximal periods in the pulse rate of the mother which looked like some humoral effect (diffusible) controlling the rates of heart-beat of both mother and fetus. It was observed that stimulation of the vagus in the mother did *not* alter the heart rate in the fetus. The temperatures of the rats are not given.

Vascular reflexes. The development of vaso-motor reflexes in the dog and cat has been studied mostly by G. A. Clark (1934) at Sheffield.

The fetal blood pressure is susceptible of considerable changes. With every uterine contraction there is a disturbance of the arterial

pressure of the fetus which apparently becomes less marked as the age of the fetus advances. This observation suggested a vaso-motor reflex, but in Clark's opinion the cause is otherwise and is due to the cutting down of the fetal blood supply consequent upon the contraction of the uterus interfering with the placental circulation. In Clark's view vaso-motor reflexes are not established till after birth. Cardio-aortic and carotid sinus reflexes appear to be developed in puppies 4 to 6 days after birth and in kittens about the 11th day. Pressor reflexes are developed earlier and have been seen 3 to 4 days after birth. Here again we would repeat that the date of appearance of such a phenomenon as a vaso-motor reflex may be very differently related to the date of birth in different animals.

The exchange of gases between the fetus and the mother. The data required for a complete exposition of the exchange of gases in the placenta are the following:

1. The rates of blood flow in the fetal and maternal circulations.
2. The percentage saturations with oxygen and the CO_2 contents of the blood reaching and leaving the placenta.
3. The oxygen and CO_2 dissociation curves.

There is no animal about which all this information can be given, and it is a case in which the reader should be warned against the application of information obtained upon one animal to patch up discrepancies in that obtained from another—for it is not clear that the mechanism of placental respiration is the same in all mammals, indeed it seems clear that it is not so.

Let us consider the items set forth above in order.

The blood flow in the mother and the fetus. The only animal in which we know much about the maternal blood flow is the rabbit, and even in the rabbit it is not the blood flow through the cotyledons which has been investigated but that through the uterus. The results in the rabbit are very striking. The period of gestation is 30 days more or less. Up to the 20th day there is a rapid increase in the quantity of blood flowing through the uterus, after which the stream swells but little and after the 22nd day remains constant. At the 20th day the embryos are very small, perhaps 3 grams weight apiece. The picture is therefore clear: first the maternal blood flow is established, adequate for the needs of the embryos up to birth and then the embryos grow rapidly.

The points given in table 12 are taken from a curve based on experiments by Barcroft, Herkel and Hill (1933), to which some as yet unpublished results have been added.

It follows inevitably that in the rabbit, since the oxygen consumption must be more or less a function of the size of the fetuses, there must after the 20th day be an ever increasing demand for oxygen on a relatively constant supply of blood. A demonstration of this was given by Barcroft, Flexner and McClurkin (1934). The method of experiment was as follows: the ovary on one side was excised and after recovery the doe was mated; the uterus on the operated side contained no fetuses. Samples of blood were withdrawn by means of a hypodermic syringe from the vein coming away from the uterus, on each side. The contrast between the bloods from the pregnant and the non-pregnant sides may be judged from the figures (table 13) taken from a curve representing the results. It seems then as though the pregnant

TABLE 12

Fetal age, days	14	18	20	22	24	26	28	30
Blood flow through uterus, cc. per fetus per minute	0.6	1.7	2.2	2.6	2.9	2.9	2.9	2.7
Approximate weight of one fetus in these experiments, grams	<1	1.5	3	7	12	18	26	<40

TABLE 13

Average percentage saturation with oxygen of blood coming from uterus

Days of fetal life	6	10	14	18	22	26	30
Pregnant side	80	80	75	70	50	40	28
Non-pregnant side	60	60	60	65	67	68	69

side first showed signs of hyperemia yielding, in the absence of any great demand for oxygen, venous blood which is highly saturated with oxygen, whilst in the late days of pregnancy the saturation drops to a low figure. On the non-pregnant side the saturation remains remarkably steady, the tendency being to rise as pregnancy advances.

It is evident that the possibilities of acquiring oxygen are exploited in a high degree by the fetus and we are left to wonder what can be the respiratory conditions in the case of does in which parturition is delayed by the administration of Prolan B and in which the young apparently continue to grow in the womb to a size much beyond that at which they are born in the usual course of events.

Putting aside the embryo itself, it would be difficult to find an in-

stance of such reduction of the blood as occurs in the uterus at the end of pregnancy in the rabbit; it is not surprising therefore to find a special and very beautiful mechanism by which this reduction is effected. So far as is known this mechanism is confined to the rabbit, but it may also occur in other and perhaps smaller mammals in which the period of gestation is short and the rate of growth rapid. The mechanism which was demonstrated by Mossman (1926) is as follows: In the placenta the capillaries of the fetal circulation, in essence, lie parallel to and in intimate contact with those of the mother, but the streams run in opposite directions; the blood which enters the placenta from the fetus finds itself in touch with the maternal venous blood about to leave the placenta. The tension of oxygen in the latter is very low, but the tension of oxygen in the blood entering from the fetus is even lower so the fetal blood commences to pick up oxygen on its entry into the placenta and goes on picking up oxygen all the way along its course, leaving the placental capillary highly oxygenated. It can in fact leave the placenta bearing oxygen at a tension only just lower than that of the maternal arterial blood. I have stressed tension rather than percentage saturation because, as we shall see later, the hemoglobin in the fetus is peculiar; over most of the dissociation curve it is more highly saturated at any given oxygen tension than that of the mother. Therefore at most points on its course through the placental capillary, the fetal blood might conceivably be more highly saturated than the adjacent blood in the mother even though the oxygen tension at the point in question was lower in the fetal than in the maternal blood.

This system of vivi-diffusion does not appear to obtain in the larger mammals; in them the fetal capillaries either course along the surface of, or actually dip into the considerable sinuses through which the maternal blood flows. The following scheme representing the condition under which fetal respiration takes place was put forward by Dr. W. H. Newton (1935). Imagine the maternal blood coursing through a sausage (representing the sinus), arterial at one end and venous at the other. Imagine that through this sausage the fetal capillaries run transversely. The blood in those which cross at the arterial end can leave highly oxygenated, whilst the blood in the fetal capillaries at the venous end must leave with an oxygen tension lower than that of the maternal blood leaving the placenta. The blood from all the fetal capillaries mixes with the result that whilst the oxygen tension in this mixed blood must be considerably lower than that of the maternal

arterial blood, it may yet be appreciably higher than that of the blood in the uterine veins.

With regard to the degree to which the maternal blood becomes reduced in the placenta, there is unfortunately no series of observations at all comparable, either in number or completeness, with those on the rabbit which we have already discussed, and at present evidence is lacking, though it may in future be forthcoming, of a progressive reduction of the maternal placental blood as pregnancy proceeds.

It is very difficult to obtain satisfactory samples of the venous blood leaving the cotyledons in the goat or the sheep. The vessels are very small; that difficulty is surmountable by the use of delicate technique. The real trouble lies in the amount of manipulation to which the uterus must, or at least may be subjected, before the samples are obtained. In order to obtain samples of blood from the umbilical vessels, the uterus must be exposed and an incision made through which the fetus is withdrawn. This is bad enough, but to obtain blood from the cotyledons, the uterus, or the portion of it in question, must be turned inside out and its surface subjected to considerable manipulation in order to expose the delicate veins which are required and to be sure that they are emerging from the cotyledons and not from the muscle. The reaction of the uterus to such treatment appears to be a general contraction which tends to cut down the blood flow and thus render the maternal blood much more venous than it otherwise would be. It is of course easy to obtain blood from the general body of the uterus in the goat or sheep, but the assumption that this can be regarded as placental blood does not seem to be justified.

On a few occasions I have obtained samples which seemed to me satisfactory, having regard to agreement between duplicates and the general facility with which samples were taken, and these I have used, but I have not so far obtained a satisfactory series. The caution with which this subject must be approached is shown by a pair of analyses obtained from a goat which had twins in the uterus; from a cotyledon on one side I obtained blood which was 69 per cent saturated; later, from a cotyledon on the other, the blood was only 30 per cent saturated.

The analyses given in table 14 seemed to be technically satisfactory.

These results cover only the middle portion of fetal life, though in it there was a fourfold increase in the fetal weight, yet during that period there was no indication of a falling off in the percentage saturation of the maternal placental blood. Evidently therefore over this period the maternal supply of blood kept pace with the demands of the fetus.

During the middle of fetal life therefore in the goat we may take it that the fall in saturation of the maternal blood in the placenta is from about 95 per cent saturation to about 60 per cent saturation.

The saturation of the blood in the umbilical vein. Though the experimental difficulties encountered in obtaining blood from the placenta apply in a less degree to the winning of samples from the umbilical vessels, yet these vessels present a difficulty of their own, namely, that any considerable degree of manipulation causes them partially or completely to close up, thus restricting the flow of fetal blood through the placenta and presumably altering the gaseous content of the blood in the umbilical vessels. The samples must be taken rapidly and with a minimum of manipulation, though it is probably best to cut open the integument of the cord and lay bare the vessel.

TABLE 14

SERIAL NUMBER OF GOAT	FETAL AGE	WEIGHT OF FETUS	PERCENTAGE SATURATION WITH OXYGEN OF MATERNAL BLOOD LEAVING PLACENTAL COTYLEDON
	days	*grams*	
8	85	278	49
10	101	646	64
11	106	690	62
3	115*	1195	69 (1)
		1250	55 (2)
Sheep 2	58	251	66

*Twins 1 and 2.

The literature of this subject is given by Schlossmann (1932) in his excellent article in *Ergebnisse der Physiologie.* Eastman (1932) has obtained a very consistent series of analyses of blood from the umbilical vein at birth in 15 cases; on the average he obtained a saturation of approximately 50 per cent in this vessel and 16 per cent saturation in the umbilical artery. In his experience the saturation of the blood supplying the fetus *in utero* is 63 per cent (in a single case) and of that in the umbilical artery 30 per cent. Suffice it to say here that most of the records of oxygen in the blood of the umbilical vein have given very low results, the striking exceptions being those of Bidone (1931).

In the sheep the readings we have obtained for the saturation of the blood in the umbilical vein have been fully consistent until the 137th day when low readings commenced to appear (table 15), but in particular attention may be drawn to no. 29 (fetal age 144 days) in

which a reading of 49 per cent was obtained near the commencement of the experiment and a reading of 70 per cent some quarter of an hour later. There seemed no reason to doubt the correctness of either reading, nor was the fetus "going down the hill," for the low reading was the earlier of the two, and it seems likely that this and others like it at the end of pregnancy are due to tension—perhaps a passing tension—of the uterine musculature causing it to seize the maternal vessels and restrict the flow of maternal blood.

TABLE 15

SERIAL NUMBER OF SHEEP	FETAL AGE	PERCENTAGE SATURATION WITH OXYGEN OF BLOOD FROM	
		Umbilical vein	Umbilical artery
	days		
8	98	69	38
50	101	84	49
11	111	79	
		82, 86 later	64
14	112	83	65
		82	
16	124	79	23
17	126	76	46
19	137	42	10
27	138	75	43
29	144	49	18
		70 later	

The actual oxygen contents of many of these samples are to be found in table 11.

In a personal communication, for which we are indebted to Prof. H. Knaus (1934), he draws attention to the increased tone and contractability of the uterus during the last few days of pregnancy.

Over most of pregnancy the saturation was about 80 per cent.

The results given in table 16 were obtained in goats, with less good technique; they are more irregular but the highest readings, which are most likely to be correct, agreed with the above.

I have given these results in some detail because they definitely present a different picture from that portrayed by most of the earlier workers, the exception being Bidone. The whole trend of the earlier observations, most of them upon man, have tended toward the impression that the oxygen content of the blood going to the fetus is very

low. The above figures point in precisely the opposite direction; while of course it is markedly lower than that of normal arterial blood (95–90 per cent saturated) it is still much higher than that of normal venous blood. It is perhaps worth pausing to consider the issue, which is doubtless of great importance to the fetus. There is this difference between the above experiments and those of the earlier workers, namely, that the latter were nearly all at a very late stage in fetal life, whereas the above experiments range over the last half, or more than the last half of the period of gestation. Even then in earlier experiments I sometimes obtained low results but with practice and improved technique I have ceased to do so and indeed I think it is fair to say that I only do so when my technique has gone obviously wrong and probably I could do so at will! But with experiments at a late stage of preg-

TABLE 16

SERIAL NUMBER OF GOAT	FETAL AGE	FETAL WEIGHT	PERCENTAGE SATURATION WITH OXYGEN OF BLOOD FROM	
			Umbilical vein	Umbilical artery
	days	*grams*		
7	79	167	53	15
10	101	646	60	
11	106	690	81	
12	113	1255	77	
22	144	2280	84	27
			49 later	30

nancy it is different. At one time I supposed that they always gave low results but experience and the multiplying of experiments has shown that this is not the case. The most variable results may be obtained, sometimes high, sometimes low, even in the same animal, e.g., goat 22 and sheep 29, at different times. My belief is that in these animals the low readings were due to passing contraction of the uterine muscle which cut off the maternal blood supply. How far this hypertension in the uterus was a natural event at this stage of pregnancy and how far it was due to experimental manipulation must be left for future work. Certainly in rabbits when I have been measuring the blood flow through the uterus shortly before the birth of the fetuses, I have seen a remarkable restriction of the maternal blood stream associated with rhythmic contractions of the uterus.

This is perhaps the place to pay a tribute to a series of most inter-

esting and important papers by Haselhorst and Stromberger (1930, 1931). They have made numerous observations on the blood gases in the umbilical and other vessels in man; some of their results will be discussed later, but in the present connection, the point is that these authors obtained markedly low results for the oxygen in the blood of the umbilical vein during Caesarean sections, results considerably lower than during normal births. They believe that the increased oxygen saturation during normal births is due partly to a faster flow of blood through the uterine sinuses and partly to a slower flow through the fetal capillaries. This is clear: in small animals in which the colour of the blood in the umbilical vein can readily be seen, the blood is bright red if a Caesarean section is rapidly and cleanly made—but the colour soon becomes darker.

The saturation of blood in the umbilical artery. There is little to be said further than what has already been pointed out, namely, that the saturation is very low, varying apparently within considerable limits, say between about 45 per cent and about 15 per cent.

The transfer of oxygen from the maternal to the fetal blood. The analyses already given have shown that over a period covering most of pregnancy, the fetal blood leaving the placenta is about 80 per cent saturated whilst the maternal blood leaving it is perhaps 66 per cent saturated.

The earlier observations as to the mechanism of the transfer of oxygen from the maternal to the fetal blood are discussed in Schlossmann's article. While there seemed little doubt in the light of the work of Huggett (1927) that diffusion accounted for the passage of oxygen across the placental membrane, the work of Haselhorst and Stromberger shed quite a new light on the mechanism by which this could take place. They made two important observations, first, that at birth the blood in the umbilical vein may have a higher oxygen saturation than that in the uterine vein; and secondly, that the oxygen dissociation curve is situated "more to the left" (that is to say, that the blood has a higher affinity for oxygen) in the fetus than in the mother. The first of their observations has been amply confirmed by us on sheep and goats over a great part of the period of pregnancy; the second is also true, or at least true in the main. The point to which we shall now direct ourselves is the mechanism of this disparity in the dissociation curves in virtue of which oxygen can at once pass from the place of higher to that of lower partial pressure whilst at the same time it may be passing from the place of lower to that of higher saturation. When I say that

the generalisation of Haselhorst and Stromberger is true in the main, the reservation which I wish to imply is this: the curves at their upper parts, say at about 90 per cent saturation, may cross; they seem to do so in the goat. This possibility was not evident to Haselhorst and Stromberger because their dissociation curves were computed from rather few data by means of Hill's equation, assuming a value for n in that equation common for the fetal and maternal bloods. In the nature of the case the curves therefore could not cross. I wish to make it clear that I am not critical of the technique of these authors. There is nothing but praise for their progress in a field where the opportunities offered were so restricted and indeed I feel a little responsible for their method of computation as in former years I had carefully instructed my old colleague Stromberger in that method of treating results. But when we had confirmed their main result on goats, from which animals we could obtain abundance of blood and with sufficient workers available for the purpose, it did seem worth while actually to trace out the dissociation curves point by point. The results are given in detail by Barcroft, Elliott, Flexner, Hall, Herkel, McCarthy, McClurkin and Talaat (1934). Here also should be mentioned the work of Eastman, Geiling and de Lawder (1933), who, in man, determined the dissociation curves of the mother and fetus at birth.

As regards the fact that the blood of the fetus has a greater affinity than that of the mother for oxygen, over the physiologically important part of the curves, all are agreed. About the cause of the difference, the opinions as expressed have been more at variance, though the difference may be capable of explanation. The Cambridge workers and those at Baltimore are agreed that the curve of the mother is displaced as compared with the normal and has a less affinity for oxygen than the normal; there is good reason for attributing this change to an alteration of the hydrogen ion concentration in the blood; on this both parties are agreed.

Carbonic acid transport. Whilst on the subject of hydrogen ion concentration a diversion may be made to say a few words about CO_2 transport. The literature will be found in the papers of Eastman, Geiling and de Lawder and of Keys (1934). The latter author, by two methods, has calculated the CO_2 pressures in the fetal and maternal circulations and confirmed the views of previous authors who held that diffusion could account for the passage of carbonic acid from the fetus to the mother.

The work of Keys indicates that the fetal plasma has an alkali re-

serve definitely higher than that of the mother though lower than normal. The question arises how is this difference maintained? To that there is no certain answer, but it may or may not be associated with the observation of Roughton and his colleagues (1935) that the fetal blood is deficient in carbonic anhydrase.

Fetal hemoglobin. Where there seems to be less unity is with regard to the fetal curve. Eastman, Geiling and de Lawder point out that this curve does not differ except in detail from that of Bock, Field and Adair (1924) for a normal man. That contention may be quite consistent with an appreciable departure from the normal curve of the woman who bore the child. The fetal curves of our goats show a tendency to cross the maternal normal curve at a high oxygen saturation. This is more marked in most of our curves than in that of Eastman, Geiling and de Lawder, though in our series by the time the fetus is ripe for birth, the condition of affairs is much as shown by the above authors.

The divergences of the fetal curve from normal, and the fact that these could not be explained on the ground of abnormal alkalinity suggested an investigation of the properties of fetal hemoglobin. The work was divided into two sections, one dealing with solutions comparable in concentration with blood, the other dealing with dilute solutions susceptible of spectroscopic analysis. The first series was undertaken by McCarthy (1933) who found that the hemoglobins of the mother and the fetus were different, that of the fetus taking up oxygen more readily than that of the mother, whilst 5 weeks after birth no difference could be detected between the hemoglobin of the kid and of its mother. Hall (1934a, 1934b) working on the maternal and fetal hemoglobins of goats of the same series obtained results similar to McCarthy and extended the field not only to rabbits but in addition he found that the hemoglobin of the chick embryo and indeed of the hatched chick took up oxygen more readily than that of the mother. Indeed in no form of life at present studied have the fetal and maternal hemoglobins been identical but man presents an interesting anomaly. Haurowitz (1935) first shewed, and it has been confirmed by R. Hill (1935), that human fetal hemoglobin has a less affinity for oxygen than that of the mother. How then Eastman, Geiling and de Lawder's curves? The results of Haurowitz and R. Hill were obtained on dilute solutions of hemoglobin; if suspensions of maternal and fetal corpuscles in identical buffer solution are used instead, the affinities are reversed and at a given oxygen pressure the fetal blood is more highly saturated

than the maternal, just as in the case of the blood studied by Eastman and his colleagues.

Naturally great interest attaches to the transition from the fetal to the maternal hemoglobin which takes place at birth: but the happenings associated with birth are outside the scope of this article.

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THE NATURE OF BACTERIOPHAGE AND ITS MODE OF ACTION

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Despite the passage of some twenty years since the discovery of bacteriophage it must be admitted that three major questions concerning it remain unanswered. These are: 1. What is phage? 2. How does it act upon bacteria? 3. What is the biological significance of its ubiquity? That the biologists' inability to furnish an exact definition of what phage is, to analyze with precision the essential mechanism of the bacteriophage-bacterium reaction, or to fathom the rôle played by phage in nature, is not an indication of neglect, is evidenced by a literature comprising several thousand titles. Rather it signalizes the peculiar difficulties which always seem to attend the investigation of complex biologically active materials whether these be hormones, enzymes, antibodies, or viruses. Further it does not detract from the value of the work already done; this work has served to define the field, establish general relationships and solve many of the important primary problems. To it has been brought the best of modern bacteriological and immunological techniques. Nevertheless, it seems to the writer that future progress demands not further extension of the orthodox bacteriological viewpoint but the application of the powerful technical and theoretical aporoaches of biochemistry. The day of what may quite properly be called the qualitative approach is drawing to its close and phage investigation is already benefiting to a steadily increasing degree from utilization of the methods of general physiology. The present review is a cross section of a transition period; if it were being written a few years hence more positive answers to many moot points undoubtedly would be forthcoming.

"Bacteriophage" or more simply "phage" is a generic term including a large group of agents which share in common the ability to produce dissolution of growing bacterial cultures (lysis); they all possess the curious property of regenerating themselves during contact with growing susceptible bacteria. For any given phage both the lytic capacity and the power to propagate are sharply limited to definite bacterial

substrates (specificity), and these limits vary widely between phages. Phages pass through bacteria-retaining filter candles and consequently were long considered to be sub-microscopic although recent work has indicated that some at least are within the lower range of microscopic visibility. They are widely distributed in nature and are found normally in the intestinal tracts of man and animals.

THE PARTICULATE NATURE OF PHAGE. In his early work d'Herelle suggested that phage exists in the form of corpuscles, citing two major experimental facts to uphold his contention. In the first place, dilutions of phage spread upon a bacterial substrate produce phage "colonies" or plaques; each plaque is interpreted as being the locus of action of one phage particle and their number is roughly proportional to the amount of phage originally used. In the second place, if identical amounts of serial dilutions of phage are added to broth cultures of susceptible organisms all the cultures up to a certain point will undergo lysis and each lysed culture will contain phage of normal titre. Beyond this dilution end point the tubes will not show lysis nor will there be any production of phage in these cultures.

Not only do these facts indicate that phage is particulate but they offer two means of determining phage quantitatively—the so-called plaque count method and the serial dilution method. The plaque count is analogous to the bacterial plate count, the growth substrate being the film of susceptible bacteria instead of a nutrient agar. In the serial dilution technique one actually determines the minimal phage dilution capable of producing lysis under certain set conditions—a quantum not necessarily identical with the phage particle itself (Krueger, 1932 b). Both procedures are open to serious practical as well as theoretical objections and do not lend themselves to accurate quantitative work (Krueger, 1932 b; Bronfenbrenner and Korb, 1923–24; Bronfenbrenner and Korb, 1925 a; Doerr and Zdansky, 1923; Nakamura, 1923–24). Clarke (1927) undertook a theoretical consideration of the serial dilution method of phage titration and showed that when the dilution constant is 0.1 only 60 per cent of parallel runs should give identical end points if phage is particulate. His analysis of Bronfenbrenner's titration data indicated that in actual practice the end points were identical in 85 per cent of parallel runs. Clarke suggested no explanation for this discrepancy between theoretical and experimental results. More recently Feemster and Wells (1933) determined statistically how many tubes in a large series would fail to receive a phage particle if various dilutions of phage are added to broth cul-

tures of susceptible organisms. The results were in close agreement with the laws of chance distribution of particles in suspension, substantiating further the particulate concept.

Early observations on the ultrafiltration of phage preparations resulted in the diameter for phages as a group being fixed at approximately 20 $\mu\mu$ (Bechhold and Villa, 1925–26; Prausnitz, 1922; Stassano and de-Beaufort, 1925). Von Angerer (1923–24) obtained about the same figure from a study of the turbidity of phage preparations. Bronfenbrenner (1927) has questioned the validity of conclusions based on the type of experiments cited because of his own findings that the colloidal particles present in filtrates are not at all uniform in size and further do not represent units of the active lytic substance, but rather aggregates to which the active material is attached in some way. In his experiments phage particles were deposited on an ultrafilter and were washed several times with water. The filtrates contained no active phage; however, if the particles were washed with broth phage appeared in the filtrate (Bronfenbrenner, 1927). Krueger and Tamada (1928–29) furnished further experimental evidence of this concept; they found that a purified protein-free phage would pass through an ultrafilter which ordinarily retained the crude phage. Hetler and Bronfenbrenner (1931) have again investigated the same point using the classical diffusion coefficient method of Northrop and Anson (1929). They conclude that a phage preparation contains particles of varying size and that the active principle is adsorbed on these vehicular particles, the phage itself having a very small diameter of not more than 0.4 $\mu\mu$.

Somewhat indirect support of the idea that the active lytic principle is associated with some sort of vehicle or carrier is contained in the observations of Krueger and Tamada (1929), Beard (1929), Kligler and Olitzki (1931 c) and Kligler, Olitzki and Aschner (1931 b) who report the increased susceptibility of phage in purified preparations to chemicals, heat and simple change in pH. These results would be anticipated if phage is borne by some inactive carrier, the latter acting as a protective colloid. It very well may be that the carrier particle represents a bacterial fragment formed by cellular disintegration during lysis. The protection of the lytic principle by the carrier is then analogous to the well known protection of an enzyme by its substrate (Euler and Laurin, 1919–20) during exposure to heat and chemicals.

Diametrically opposed to the concept of an active principle carried on an inert particle are the experiments of a number of competent workers. Elford and Andrewes (1932) have studied carefully the problem of

particle size using a comprehensive group of phages and a carefully controlled ultrafiltration technique. Among their phages the particle size ranged from approximately 10 $\mu\mu$ for dysentery phage to a maximal size of 75 $\mu\mu$ for several phages. The particles in any single phage preparation were apparently of uniform size regardless of the bacterial substrate employed in their propagation. Furthermore, they could detect no difference in the particle size of purified preparations and ordinary lysates. Evidence supporting their work has been obtained by the use of Barnard's method of microphotography employing monochromatic ultraviolet light. With this technique Burnet (1933 a) secured photographs of granules having a uniform diameter of about 50 $\mu\mu$ in concentrated phage preparations. Schlesinger (1932 b) determined the particle size of phage by a different technique, a modification of the Bechhold-Schlesinger method suitable for determinations on substances which leave no sediment on prolonged rapid centrifuging. He deduced from the rate of sedimentation a particle size of about 80 $\mu\mu$ for the phage studied. He also produced phage (1933 a) from cultures grown on a synthetic medium and found that the cleared culture gave a well defined Tyndall effect. In the ultramicroscope fitted with an azsimuth diaphragm of Siedentopf and Szegvari, the particles in the phage filtrate were of uniform size and brightness. Since the particles faded gradually without twinkling as the screen was narrowed, Schlesinger concludes that the particles are spherical. There was an approximate agreement between the ultramicroscopic count and the number of plaques produced on a bacterial substrate, the biological titre being between 55 per cent and 80 per cent of the direct count.

It may be concluded from the work cited that phage in ordinary preparations is in colloidal suspension. Various phages differ in particle size, the range in general being from 10 $\mu\mu$ to 75 $\mu\mu$. It is probable that the extent of variation in particle size in any given preparation is less than had been assumed previously. There is experimental evidence both for and against the view that phage consists of an active principle carried on some inert colloidal particle; this question is as yet unsettled.

The electrical charge of phage. Data on the electrical charge carried by phage particles have been obtained by observing the direction of migration in an electrical field and by adsorbing phage from lysates with positively and negatively charged colloids. For the most part the early experiments were poorly controlled; the effects of pH, etc., were entirely overlooked and the results showed little agreement.

Todd (1927) carried out some careful experiments on the electrophoresis of Shiga phage. He found that migration occurred towards the anode over a range of pH 3.6 to pH 7.6. Krueger, Ritter and Smith (1929), working with several anti-coli phages and with two types of cataphoresis apparatus, found that the phages were negatively charged from pH 9.0 to pH 3.4. Below this point the particles assumed a positive charge. Later Kligler, Olitzki and Aschner (1931 b) performed cataphoresis experiments with ordinary coli-phage and with purified protein-free phage. Their results showed that phage within the pH range 4.0 to 12.0 carried a negative charge; the same phage in a pure state was in general negatively charged in neutral and slightly alkaline solutions but became positively charged as the hydrogen-ion concentration was raised. They conclude that protein present in ordinary phage preparations affects the results of cataphoresis experiments and they feel that previous experiments are open to criticism for this reason. Burnet and McKie (1930 b) studied the cataphoretic migration of twenty different phages. They found that the phage particles carried a negative electrical charge in all cases except one. Using apparatus modified from that of Todd, Natarajan and Hyde (1930) carried out electrophoresis experiments with several phage races. Over a fairly wide pH range they observed migration towards the anode while with two phages amphoteric behavior was noted in slightly alkaline solution.

The experimental data then indicate that phages generally, with only infrequent exceptions, bear a negative electrical charge within the limits of pH 3.4 to pH 12.0. Below pH 3.4 the charge tends to become positive and measurements are made difficult by the inactivating effect of H^+ ions on the phage. Amphoteric behavior in alkaline solutions has been observed occasionally; there is some evidence that protein-free preparations differ from crude lysates in electrophoretic behavior.

THE EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON PHAGE. *A. The effect of temperature.* From the early experiments on heat inactivation of phages it became evident that there was a considerable degree of variation in susceptibility to heat between various phage races. However, much of the older work was lacking in controlled experimental conditions and important factors were often over-looked. There is no doubt, however, that phages in general are inactivated completely by exposure to temperatures of 70° to 75°C. at pH 7.4, for 30 to 60 minutes. Instances of exceptional heat resistance have been recorded by Sertic and Bulgakov (1931) and Rakieten (1932). Nanavutty (1930) showed experimentally that the heat resistance of phage varies with the sus-

pending media. In plotting his data as logarithms of surviving active substance against time of exposure the curves tended to become concave; Nanavutty interpreted this as indicating variations in the capacity of individual particles to resist heat. Krueger (1932 a) reported on the heat inactivation of staphylococcus phage over the temperature range 51°–62°C. using an accurate activity method for the titration of surviving phage substance. The process of inactivation at all temperatures studied followed the course of a monomolecular reaction and the velocity constants for the various temperatures used gave a good fit in the Van't Hoff-Arrhenius equation. The critical thermal increment was about 101,000 suggesting that the chief reaction involved in the heat inactivation of phage is a protein denaturation. This is supported by Vedder's observation (1932) that dried preparations are more thermoresistant than fluid ones; proteins in general resist denaturation best when dry. Dreyer and Campbell-Renton (1933) also found the rate of deterioration by heat to be adequately expressed by the usual equation for a monomolecular reaction. Kligler and Olitzki (1931 c) showed that "purified" protein-free phage is more susceptible to heat than are ordinary impure preparations. Bronfenbrenner (1932) found that it was possible to protect to some extent against heat inactivation by the use of calcium chloride. Employing the substances which Spiro used as denaturation preventives, for example glycerine and carbohydrates, considerable phage activity could be preserved even on exposure to 70°C. Krueger and Scribner (1935) have more recently studied the effect of pH on heat inactivation of phage; in their experiments both H^+ and OH^- ions were found to catalyze the inactivation process. For example, the percentages of total original phage surviving exposure to 57°C. for 0.5 hour at various H^+ ion concentrations were respectively pH 7.5 = 50 per cent, pH 9.0 = 1 per cent, pH 6.0 = 32 per cent, pH 5.75 = 3 per cent. They point out that these results are analogous to the catalyzing effect of H^+ and OH^- ions on the heat inactivation of many enzymes.

Burnet and McKie (1930 a) showed that the heat inactivation of phage which occurs readily at 60°C. in the presence of sodium, potassium and ammonium salts is partially or completely prevented by the addition of small amounts of calcium, magnesium or barium salts.

From what has been said it is evident that with certain exceptions the majority of phages are readily destroyed by exposure to moderately high temperatures for any considerable period of time. The inactivation process follows the course of a monomolecular reaction and has the

high critical thermal increment uniquely characteristic of protein denaturation, suggesting that the inactivation involves protein denaturation as an important phase of the phenomenon. The composition of the suspending medium exerts a marked influence on the thermolability of phage.

d'Herelle (1926) reports that "young" phage resists exposure to temperatures of liquid air (−180°C.) for 10 minutes, while older phage is destroyed by this treatment. Rivers (1927) studied the repeated freezing (liquid air) and thawing of phage and found that the loss of activity due to such treatment is influenced by the composition and concentration of the medium and that in general it is comparable to the effect of similar treatment on complement, trypsin and colon bacilli.

B. The effect of ageing and drying. d'Herelle (1926) has emphasized the capacity of phage to preserve its activity even after nine years in sealed ampoules. On the basis of plaque counts he found reduction of active particles occurred so that the survivors numbered from 2 per cent to 4 per cent of the original concentrations. In his experiments the capacity for survival was greatly influenced by the bacterial substrate used in preparing the phage suspension. Resistance to ageing varies considerably among phage races. Marshall and Paine (1931) have reported that phage may survive at least eight years in sealed ampoules without changing its chief characteristics. Martin (1930) records the satisfactory keeping qualities of a dysentery phage for twenty months.

d'Herelle (1921 a) found that phage preparations would retain some degree of activity even after complete drying. Vedder (1932) records the preparation of active phage preparations in desiccated form from bouillon filtrates.

C. The effect of irradiation. Among the earlier experiments on the inactivation of phage by irradiation may be mentioned those of Appleman (1922) who found that the activity of phage was completely lost after exposure to ultraviolet light for ten minutes. Zoeller (1923) confirmed Appleman's findings; Gildemeister (1922) concluded that the susceptibilities of phage and bacteria to ultraviolet light were comparable. The observations of Gerretsen, Gryns, Sack and Söhngen (1923–24) are not in accord with these results. They exposed a phage for B. radicicola to a Heraeus lamp at 30 cm. distance and found that there was no demonstrable effect after 30 minutes' exposure. Some active material was found after 2 hours of treatment and the activity was lost altogether only after 2½ hours. Mizuno (1929) also experimented with destruction of phage by ultraviolet light. He showed that the effect

of the rays is inversely proportional to the depth of the medium and that the type of medium greatly influences the results. Broth exerts a protective action by absorbing the rays. Gates (1934) has studied carefully the inactivation of a Staphylococcus aureus strain and its homologous bacteriophage by the various wave lengths of the quartz mercury vapor arc between 238 and 302 Angstrom units. Throughout this range the incident energies required to kill the staphylococcus and to inactivate the phage run strictly parallel although the phage readings are at a higher energy level. Gates stresses the similarity in the shapes of the energy curves and concludes that in both instances absorption of the radiation occurs through the same organic structures.

Mizuno (1929) reports that phage is not influenced by exposure to x-rays. Beckwith, Olson and Rose (1930) exposed seven races of coli phage for 30 minutes at 14 inches distance from the target of an x-ray tube actuated by 75,000 volts. In three instances there was a reduction in activity of the phage but no effects were noted in the other four cases.

Brutsaert (1923 b) exposed phage to radium emanations. After 24 hour's exposure a coli phage showed somewhat diminished activity but in several instances there was no demonstrable effect on phage after prolonged exposure.

D. The effect of chemicals. Many investigations have dealt with the effects of chemicals on phage but unfortunately they have for the most part involved different technics and a variety of phage races, rendering comparison of results all but impossible and the data have been qualitative rather than quantitative. That is to say, the rate concept has been neglected and the results state that phage (in unqualified amounts) has survived a certain number of days in a particular solution but no phage was present after an additional number of days. This all or none sort of data is seldom helpful in building up a picture of the mechanisms involved in chemical inactivation and few generalizations can be made from it.

Many of the early papers dealing with the effects of acids and bases on bacteriophage did not take into account the temperature at which the experiments were carried out nor the length of exposure. Bronfenbrenner and Korb (1925 b) have reported the results of exposing phage to a temperature of 70°C. for three hours. Under these conditions staphylococcus phage was inactivated beyond the range pH 4.3 to pH 8.8, coli phage beyond pH 1.55 to pH 10.6, and phage for B. pestis caviae beyond pH 2.9 to pH 11.45. Bronfenbrenner and Korb's data supplement and make more specific the earlier findings of Eliava and

Pozerski (1921 b) and Scheidegger (1923). Muramatsu (1931) records observations in which phage resisted exposure to H^+ ion concentrations corresponding to pH 4.0 to pH 11.0 with little loss in activity.

Independent publications by d'Herelle (1921 a) and Proca (1927) indicate that phage can survive three days' exposure to 0.5 per cent $HgCl_2$ but is completely inactivated in four days; but little loss in potency was noted after 185 days in 1:7,000 $HgCl_2$. Prausnitz and Firle (1924) conclude that phage is somewhat less resistant to $HgCl_2$ than anthrax spores. According to Prausnitz (1922) potassium cyanide in a 2.5 per cent solution considerably reduces phage activity after three days' exposure; Borchardt (1927) (1928) states that phage successfully withstands the action of 20 per cent HCN and behaves like an enzyme in this respect. Prausnitz (1922) reports a similar effect in the presence of 2.5 per cent sodium fluoride. Kleineberger (1930 b) observed that phage will withstand bimolar NaCl solution for days without loss of activity whereas there is a considerable diminution in plaque count and activity when M/16 or M/32 NaCl is used. Phenol in 1 per cent solution destroys phage after seven days of contact (d'Herelle, 1921 a); 2 per cent lactic acid and oxalic acid (DePoorter and Maisin, 1921) or 1 per cent quinine hydrochloride (Eliava and Pozerski, 1921 b) completely inactivate phage in less than 24 hours. Six-tenths per cent formaldehyde destroys phage activity entirely within 5 minutes (Prausnitz and Firle, 1924). A number of investigators have found that undiluted glycerol rapidly inactivates phage (d'Herelle, 1920 b; Bablet, 1920; Proca, 1923). Watanabe (1923 b) working with a number of different phages observed that the resistance to alcohol varied considerably although all of them were destroyed within thirty minutes by 75 per cent alcohol. This coincides with the findings of DePoorter and Maisin (1921). Since then Bronfenbrenner and Korb (1926) and Bronfenbrenner (1926–27) have made a more detailed study of the inactivation of phage by alcohol. In their experiments purified phage preparations were much more resistant to alcohol than impure ones; this they interpreted as indicating that the inactivation was secondary to changes in the carrier particles to which the lytic principle was adsorbed.

Schultz (1928) has reported the inactivation of two anti-staphylococcus phages by trypsin in solution. Schultz and Krueger (1928–29) found that methylene blue would inactivate certain phages in high dilution. This effect was confirmed by Clifton (1930–31 b) and was proven by him to be due to oxidation of phage by photosensitized methylene blue. Perdrau and Todd (1933) extended these findings showing that

the optimal concentration of dyes is about 1:100,000 and that phage taken up by bacteria is very much more resistant to this killing effect; the inactivated phage could not be reactivated by reduction. Burnet and McKie (1930 a) found that dilute phages could be inactivated readily by methylene blue, toluidin blue and janus green. In the presence of broth and calcium salts the inactivation did not occur. In later work Burnet (1933 c) reports the interesting observation that phages belonging to the same serological group behave uniformly as regards susceptibility to inactivation by methylene blue, certain acridine dyes and urea solution. Wells and Sherwood (1933) (1934) have noted that crystal violet selectively inactivates phages for the Gram positive bacteria while it has no effect on the phages active against Gram negative organisms. They also studied the action of phenol and sodium hydroxide on phage and concluded that relatively higher concentrations of these agents as compared with the dyes, are required for inactivation and that the phages for the Gram negative bacteria are more resistant to toxic agents than those acting on the Gram positive group.

Krueger and Baldwin (1935) have more recently studied the inactivation of staphylococcus phage by safranine. The inactivation was shown to be in part photodynamic. If the precipitate which formed was dissolved by adjusting the pH of the broth 25 per cent of the total phage inactivated could be reactivated. Similarly an additional 10 per cent of the inactivated phage was obtained in an active form by adjusting the supernatant to pH 6.5. Krueger and Baldwin (1933), (1934) had previously shown that the inactivation of staphylococcus phage by bichloride of mercury follows the equation for a pseudomonomolecular reaction over most of the inactivation curve. After complete inactivation in 2.8 per cent $HgCl_2$ for 216 hours the original titre of active phage was restored by precipitation of the Hg^{++} ion. Krueger and Elberg (1934) observed a similar phenomenon in the case of inactivation of phage by KCN. In their experiments inactivation with M/650 KCN for 24 hours was quantitatively reversed by converting CN^- to $Ag(CN_2^-)$. Analogous observations, that is, inactivation followed by complete restoration of activity, have been recorded with many enzymes.

Gregorieff (1927) has found that the phage particles surviving heat, antiseptics or antiserum, produce plaques of normal size on agar; he interprets this as indicating that the survivors are not effected qualitatively—inactivation is an all or none matter and individual corpuscles differ in sensitivity to noxious agents. Krueger and Tamada (1929)

reported that phage purified by electrophoretic migration into agar and subsequent resuspension is considerably more susceptible to inactivation by chemicals and drying than ordinary phage. These observations have been confirmed and extended by Beard (1929) and Kligler and Olitzki (1931c).

E. Filtrates of bacterial cultures. Levine and Frisch (1933) (1934) (1935) have published an interesting series of observations on the specific inhibition of phage by bacterial extracts. They predicted that this phenomenon might permit classification of the salmonella group of bacteria in terms of phage action. The inhibition technic together with the absorption of phage by dead bacteria might be expected to reveal relationships which could be anticipated from the serological viewpoint but were not demonstrable by direct phage action.

They found in a study of three phages, Shiga, suipestifer and paratyphosus B races, that the bacillary extracts of the homologous strains specifically inhibited their own phages. Aertrycke extract showed a slight effect in inhibiting Shiga phage. The paratyphosus B phage was inhibited by extracts of all organisms containing the heat stable factors I and II of White, that is paratyphosus B, B. tidy, B. stanley, and B. aertrycke.

In experiments on the salmonella group Levine and Frisch resorted to absorption of phage by heat killed bacterial suspensions. The residual phage was tested and it was found that the suipestifer bacteria absorb suipestifer phage and in addition the suipestifer fraction of paratyphosus A polyvalent phage. The typhoid-enteritidis group and the aertrycke-paratyphosus B group absorb phage equally well. However, bacterial extracts from these groups can be differentiated by means of phage, for the paratyphosus B phage shows greater specific inhibition.

Immunization experiments followed by agglutinin absorption confirmed differences early noted within the suipestifer group. Apparently suipestifer strains no. 80 and no. 90 form one group, the American types and Kunzendorf strains a second group, and the Glasser-Voldagsen and suipestifer no. 100 (Hirschfeld) a third group.

Treatment of the inhibiting extracts with acid enhances the inhibiting power. Levine and Frisch believe this indicates that the acid treatment produces hydrolytic products in which the reacting groups are unaffected.

On the basis of earlier observations by the phage absorption and specific inhibition technic, it was demonstrated that polyvalent phages active against paratyphosus A or enteritidis strains contained at least

two qualitatively different fractions. This raised the question as to whether anti-phages might be used to detect the presence of several fractions in a phage. Immunization was effected with two polyvalent phages for paratyphosus A and enteritidis and with monovalent paratyphosus B and suipestifer phages. Two antibodies corresponding to the fractions of the polyvalent phages were produced. Both antisera possessed antibodies for the suipestifer fraction. Levine and Frisch conclude that similar specificities are shown by the reaction of phage with heat-killed bacilli and with anti-phage. The use of polyvalent phages with qualitatively specific fractions makes it possible to study anti-phage reactions in such heterologous combinations of reagents as will minimize interferring agglutinin reactions. Phages may differ radically in their composition and yet share a particular fraction in common. Burnet (1934) has confirmed the specific inhibition of phages by bacterial extracts. He has shown that the phage inactivating capacity of an autolysate is removed by treatment with homologous antibacterial serum. Autolysates of phage-resistant strains exhibit no inactivating reaction. Burnet suggests that the activity of the bacterial extracts is due to their containing those portions of the bacterial surface to which phage attaches itself before lysis. Gough and Burnet (1934) have prepared the phage inactivating agent in solutions giving no protein reactions. They consider it to be a polysaccharide which can be degraded to a haptene by alkali treatment. During the degradation inhibitive capacity is lost. In some instances the inhibiting agent has been very unstable and none of the extracts are equal in power to the intact bacterial surface.

THE PURIFICATION OF PHAGE. In 1925 Arnold and Weiss reported on the isolation of phage free from bacterial proteins. They propagated phage upon a bacterial substrate growing on very low concentrations of agar. After removing the bacteria and some of the metabolites the phage was extracted from the deeper agar layer into which it had diffused. By this means preparations containing a considerable amount of phage and no demonstrable proteins could be prepared. Weiss (1927b) precipitated the bacterial antigen from a phage lysate with lipoids of the Wassermann antigen. The phage was left in suspension and if an asparagin medium were used no broth proteins were left behind with the phage. Attempts to concentrate phage by alcohol precipitation were not successful. Krueger and Tamada (1929) employed the electrophoretic migration of phage into an agar gel to separate the active substance in phage lysates from the metabolites and broth pro-

teins. The phage is subsequently re-suspended by maceration of the agar in a suitable medium. This method has been used with satisfactory results by Elford and Andrewes (1932), Larkum (1935) and Muramatsu (1931). Krueger and Tamada reported an increase in activity of the phage obtained by purification. This observation is erroneous and has been shown by Krueger (unpublished experiments) to be due to phage in agar particles carried over mechanically during titration of the pure preparation by means of the serial dilution technic. Clifton (1930–31a) purified phage by adsorption on aluminum hydroxide followed by elution in secondary ammonium phosphate solution. In an analogous fashion Kligler and Olitzki (1931a) (1933) have used kaolin as an adsorbent and ammonium hydroxide in weak solution for elution. Spasowicz (1933) has also reported on the concentration of phage by means of adsorption. Rakieten and Hunt (1932) experimented with elution of phage adsorbed onto particles of kaolin but could not obtain satisfactory yields. Hosoya and his co-workers (1932) used a method for purification which had previously been employed by Hosoya and Miyata for the purification of toxins. Their final product was actively lytic and contained no demonstrable proteins. Schlesinger (1933b) was able to prepare phage in visible quantities using a strain of B. coli grown on an artificial medium. Concentration of the lytic substance was effected by high speed centrifugation in collodion tubes. Sediments of 0.5 mgm. to 2.0 mgm. from 2 liters of lysate were obtained in this way. The same worker (1934 a) has determined the properties of the jelly-like sediment he obtains from lysates by centrifugation. The sediment is Gram positive; slightly acid-fast; gives positive Biuret, Xanthroproteric and Millon reactions; a weak test for carbohydrates and contains some fat. Elementary analysis shows C = 42 per cent, H = 6.4 per cent, N = 13.2 per cent and P = 3.7 per cent. He concludes that the phage particle has a dry weight of 4.6×10^{-13} mgm., that it is chiefly protein and that it is probably a nucleoprotein.

Quite recently Karl Meyer and his co-workers have employed a new method for phage purification. Alkalinized phage is treated with colloidal iron and the precipitate discarded. To the acidified supernatant sodium flavianate is added followed by solid $BaCl_2$. The mixture is brought to pH 7.6, the precipitates discarded and the supernatant adjusted to pH 8.4. The precipitate which forms contains the phage. It is washed, suspended in acid water and the barium removed with Na_2SO_4. The phage remains in solution. One preparation contained 5.5 per cent N and was active in a dilution greater than $1:10^9$. In 1 per cent

solution the xanthoproteic, Greenberg phenol reaction and Molisch test were positive (Meyer et al., 1935).

The use of the term "protein-free" in connection with purified phage preparations is open to some objections. It is quite possible that the failure to detect protein by qualitative tests in the "pure" solutions obtained by certain methods is due to dilution of the original small protein content beyond the limits of the test procedure employed to detect its presence. Phage under those circumstances would be diluted to the same extent but lysates which still show activity, i.e., contain at least some phage, when diluted 10^{-12} are not uncommon and the activity limit of the preparation might well be beyond the range of the chemical test for protein.

ADAPTATION OF PHAGE. The possibility of phage adaptation to the action of injurious chemicals has been stressed by a number of workers as a critical criterion in establishing the living or non-living nature of phage. d'Herelle and others have demonstrated to their own satisfaction that phage adaptation can be readily and regularly produced. For example, d'Herelle, (1921a) has reported the effective adaptation of phage to concentrations of glycerol which ordinarily destroy it. Brutsaert (1923a) reports similar findings. d'Herelle (1922a), Asheshov (1922) and Schuurman (1925) have adapted phages to increased concentrations of hydrogen ions. The adaptation to anti-bacteriophage serum described by Prausnitz was confirmed by d'Herelle (1923), Bruynoghe and Wagemans (1923) and Prausnitz and Firle (1924). Wolff and Janzen (1922) and Schuurman (1925) report the successful adaptation of phage to chinosol. Prausnitz and Firle (1924) also produced adaptation of phage to phenol, bichloride of mercury and chloramine. Schultz and Green (1928–29) showed that during the course of serial exposures to trypsin phage becomes resistant to the enzyme.

Munter and Rasch (1925–26) were able to confirm Prausnitz' results only when his own particular phage and strain of B. dysenteriae were used. They interpreted the results not as an adaptation of the phage but as being due to the lysogenic nature of the bacterial strain employed. The adaptation of phage to increasing concentrations of H^+ ions reported by Asheshov (1922) (1924) were found to occur with one race of phage only. The Wollmans (1928) attempted unsuccessfully to adapt an anti-Staphylococcal phage to an acid medium. Beard (1931) has made a very careful study of the adaptation phenomenon using coli, dysentery, typhoid, proteus, streptococcus and staphylococcus phages. He could not demonstrate increased tolerance to chemicals or to heat.

Although the positive results recorded above have been emphasized repeatedly as indicating the living nature of phage it should be pointed out that the extent of the adaptation has been in many instances very limited and frequently was well within the limits of experimental error. Also it is a well-known fact that a given phage will display considerable variation in its behavior when tested over a period of time (Prausnitz and Firle, 1924; Asheshov, 1924). Everything considered it is the writer's opinion that clear cut evidence of phage adaptation remains to be presented.

THE MECHANISM OF THE BACTERIUM-PHAGE REACTION. Two outstanding facts were observed by d'Herelle early in his work on phage; these were that the lytic agent is regenerated during the course of its action on bacteria and that the turbid cell suspension eventually becomes clear. As d'Herelle pictured the phenomenon of bacteriophagy the first phase consists in fixation of the phage to the bacterium; the phage corpuscle then penetrates into the bacterial cell, multiplies and liberates its colony of young corpuscles when the bacterium ruptures. He stressed the importance of cellular lysis in producing phage, each bacterium serving as an incubator for the reproduction of phage, the latter not being active until the cell bursts. There is no longer any doubt that this view is incorrect for it has been shown that lysis of cells will occur in concentrated phage suspensions without occasioning any increase in the concentration of phage (Bail and Matsumoto, 1923; Matsumoto, 1923–24; Meuli, 1923; Krueger and Northrop, 1930).

The production of phage then is not dependent upon lysis; it has been shown to be conditioned by bacterial reproduction. Whenever the process of cell division is interfered with in any way as for example by reduced temperature (Krueger and Northrop, 1930) or by the maintenance of special experimental conditions (Bruynoghe and Mund, 1925; Doerr and Gruninger, 1923; Otto and Winkler, 1922; Zdansky, 1925) phage production is reduced.

For the most part investigations of the quantitative relationships between phage and bacteria during the course of bacteriophagy have depended upon either the serial dilution method or the plaque count for quantitative determinations of phage. Both these procedures have definite limitations and neither one is suitable for analysis of a dynamic system in which concentrations are changing rapidly (Krueger, 1932b). Krueger (1930) has described an activity method for the quantitative determination of phage based upon the observation that under certain standard conditions the time of lysis is a function of the initial phage

concentration present in a bacterial suspension. Lin (1934) has corroborated these findings using a different phage and a slightly different technical procedure. The unit used in the Krueger titration method is an arbitrary activity unit having the dimensions of a velocity constant (Krueger, 1930). The titration technic has been employed by the author and his collaborators for the past five years and the accuracy

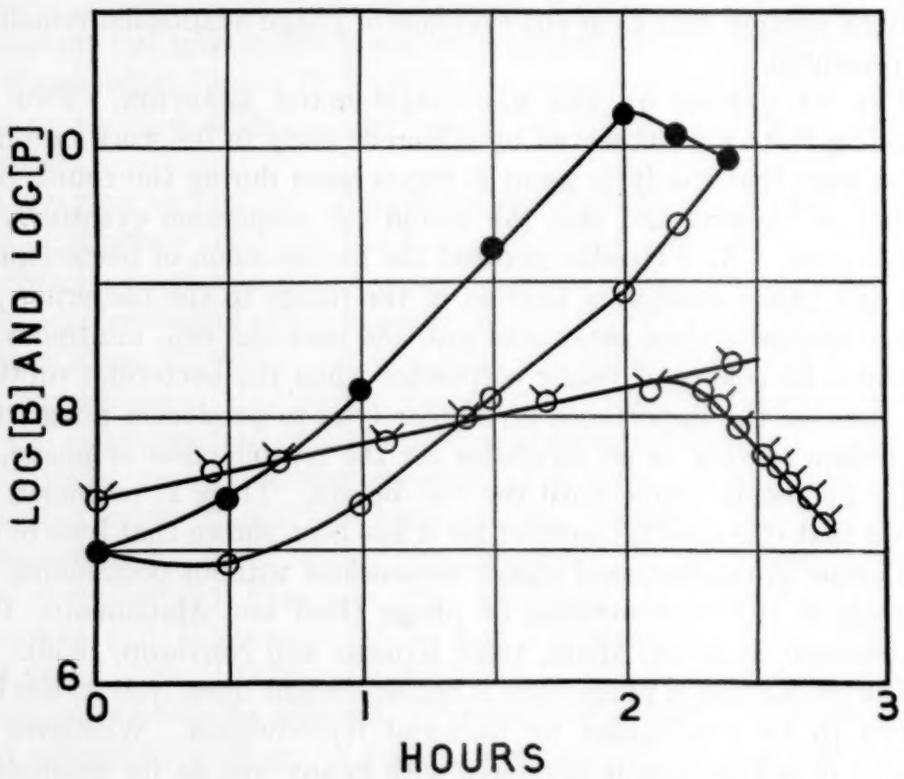

Fig. 1. Relationship of extracellular phage fraction and intracellular phage fraction to bacterial growth.

● = Total P/ml.
○ = Extracellular P/ml.
♂ = [B] in broth control.
♂ = [B] in phage-bacterial mixture.

has been shown to be well within 5 per cent. Using this titration method Krueger and Northrop (1930) investigated the kinetics of the bacterium-bacteriophage reaction. The general relationships found are shown in figure 1 in which total phage, extracellular phage and bacterial concentrations are plotted against time of sampling. It will be seen that under the experimental conditions obtaining the major portion of the phage becomes attached in some way to the bacteria. As

bacterial growth begins phage production commences and during the course of bacterial growth and phage formation the two reactions are intimately linked; any interference with bacterial growth is reflected in diminished phage production. The phage is not all within the cells but is distributed between the cells and the medium in accordance with the law of normal distribution; that is, $C_b/C_a = K$ where C_b = extracellular phage per ml. of broth and C_a = intracellular phage per ml. of bacteria. The rate of phage formation is greater than the rate of bacterial reproduction; as a consequence the ratio of phage to bacteria is constantly increasing. When this ratio reaches 125 phage units per cell (lytic threshold) cellular dissolution begins and proceeds logarithmically with time. The general relationships noted can be expressed in the form of a single differential equation $\frac{dP}{P} = K \frac{dB}{B}$ and the integral forms of the equation can be tested by comparing the calculated values for 1, bacterial concentrations at the time of lysis, and 2, the time of lysis itself, with observed experimental values. Agreement is excellent over the range studied. The equation has additional interest in predicting the relationships made use of empirically in the activity titration method (Krueger and Northrop, 1930).

In additional work on the sorption of phage by living and dead susceptible bacteria Krueger (1931) has shown that the union of phage with living bacteria is quantitatively reversible as would be predicted by the partition coefficient equation. The rate of binding was proportional to the concentration gradient and to the number of cells present. When heat-killed susceptible bacteria were employed to bind the phage it was found that equilibrium conditions could be expressed in terms of the adsorption isotherm equation and no evidence of reversal was obtained. Schlesinger (1932a) reports that about 95 per cent of the total phage is bound quickly and irreversibly by dead bacteria; a second portion is bound more slowly and reversibly while a third fraction is not bound at all.

Northrop and Krueger (1932) have attempted to make a further analysis of the phage-bacterial relationships requisite for lysis. They found that the significant condition for induction of lysis is either a concentration of about 110 phage units inside each bacterium or a concentration of about 12×10^8 activity units in each milliliter of surrounding solution. Since these two quantities are always in constant ratio to each other, as has been pointed out above, it is immaterial which one is used; they cannot be distinguished by any experimental proce-

dure carried out under equilibrium conditions. Krueger and West (1935) have shown that high dilutions of manganous salts accelerate the lytic effect of phage upon susceptible staphylococci. The time of lysis is reduced and the critical lytic threshold is lowered to about $\frac{1}{5}$ the value obtaining in the absence of manganese. The Mn^{++} ion alters the distribution of phage so that about four times as much remains outside the bacterial cells. There does not seem to be any effect on the rate of phage formation or on the rate of bacterial growth. As would be anticipated from the fact that the lytic threshold is reduced by Mn^{++} the end titre after completion of lysis in a phage-bacterial mixture is considerably less in the presence of manganese than in its absence.

In a general way this picture of the kinetics of the phage-bacterium reaction affords quantitative confirmation of much of the earlier descriptive work and in some instances states more clearly relationships which were suggested but which were not adequately investigated from the quantitative viewpoint. It was known, for example, that bacteria could be lysed in concentrated phage (Bail and Matsumoto, 1923; Matsumoto, 1923–24; Meuli, 1923) but it remained to be demonstrated that lysis is conditioned by the attainment of a certain ratio of phage to bacteria (Krueger and Northrop, 1930; Northrop and Krueger, 1932). Similarly several investigators have noted the tendency of lysates made with a given bacterium and its homologous phage to yield approximately the same final phage titre regardless of the initial phage and bacterial concentrations employed, but an explanation of the phenomenon was lacking. From the kinetic analysis of the reaction it became clear that the end titres obtained must necessarily fall within narrow limits because of the relationships existing between the rate of phage formation and the rate of bacterial reproduction (Krueger and Northrop, 1930). As has been noted above the equation for the phage-bacterium reaction predicts that the time of lysis is a function of the initial phage concentration thus furnishing a satisfactory mechanism for the empirically developed activity titration procedure. Lin (1934) has found that the general relationships established in the case of the staphylococcus and anti-staphylococcus phage by Krueger and Northrop apply also to a race of dysentery phage and the homologous bacterium.

There are several points in connection with the mechanism of phage action about which no general agreement has been reached. This is due in part to differences in the phages and bacteria used and to the variety of technical approach employed. For example, Bronfenbrenner has developed a theory of phage action in which an essential element is

the stimulation of bacterial metabolism by phage. He and his collaborators have shown repeatedly that under certain experimental conditions phage stimulates bacterial growth (Doubly and Bronfenbrenner, 1933; Bronfenbrenner, Muckenfuss and Hetler, 1927; Bronfenbrenner and Hetler, 1928; Hetler and Bronfenbrenner, 1931). On the other hand, identical growth curves in plain broth and in broth containing small amounts of phage have been reported by a number of investigators (Krueger and Northrop, 1930; Lin, 1934, Andrewes and Elford, 1932; Eaton, 1931; Bordet, 1931). Again, Burnet has objected to the use of Krueger and Northrop's data as a basis for developing a mechanism of phage production on the grounds that our information was derived from the interaction of large numbers of phage particles and bacteria and that these statistical results do not apply to the reaction between a single phage particle and a single bacterium. He has investigated (Burnet, 1929a) the early stages of phage production utilizing a single phage particle in broth cultures. His results indicate that the phage particle multiplies under some spatial constraint and that, together with its progeny, it is suddenly released when the cell ruptures. If this mechanism applies to masses of cells and phage particles as well as to single units phage production would entail simultaneous processes of bacterial growth, bacterial lysis, and phage formation. Consequently the bacterial growth curve should have a flatter slope than the control growth curve; however, there is good evidence that the growth curve in the presence of phage is either identical with (Krueger and Northrop, 1930; Lin, 1934; Andrewes and Elford, 1932; Eaton, 1931; Bordet, 1931) or steeper than (Doubly and Bronfenbrenner, 1933; Bronfenbrenner, Muckenfuss and Hetler, 1927; Bronfenbrenner and Hetler, 1928; Hetler and Bronfenbrenner, 1931) the control growth curve. In view of these facts the only mechanism by which concomitant phage formation, bacterial lysis and bacterial growth could be pictured to occur without the bacterial growth curve deviating from the control curve would involve the assumption that phage stimulates bacterial growth just sufficiently to balance the cellular deficit created by continuous lysis—a delicate balance, against the existence of which are the numerous negative attempts to detect an increased bacterial metabolism. Likewise, there exists experimental evidence indicating that lysis is not a continuous process during the time of phage formation and bacterial reproduction but rather that it begins suddenly when the critical ratio of phage to bacteria has been built up in the system (Krueger and Northrop, 1930; Andrewes and Elford, 1932).

To sum up the evidence available at the present time as it pertains

to the interaction of phage and bacteria the following points appear to be definitely established: 1. When phage and living bacteria are brought together the phage rapidly attaches to the cells until an equilibrium is established between the cellular fraction and that free in solution; this linkage to the bacteria is dissociable. 2. As bacterial growth begins, the formation of phage commences; cell division seems to be the essential conditioning factor for phage formation. 3. The rate of phage production is considerably greater than the rate of bacterial reproduction. Consequently the ratio of phage to bacteria continually increases and finally attains the critical level requisite for lysis (lytic threshold). 4. These relationships may be represented in the form of a simple differential equation the integral forms of which give values agreeing satisfactorily with those determined experimentally.

It was known empirically, as the basis of the activity titration method, that the time of lysis under standard conditions is a function of the initial phage concentration. This relationship is stated by a derivative of the primary equation which thus receives further experimental support.

THE EFFECTS OF PHAGE ACTION ON THE BACTERIAL SUBSTRATE. *A. Effects of phage on cell morphology.* Early in his work on phage d'Herelle described the changes produced in bacteria by the action of phage as he observed them under the microscope (1921c, 1926). He noted the production of elongated and spherical forms and the swelling which occurred in some instances; further he observed certain refractile granules inside the swollen cells; these he thought to be phage corpuscles whose development caused the cellular swelling. Not everyone has agreed with d'Herelle as to the frequency of bacterial swelling during phage action nor for that matter as to the significance of the swelling sometimes observed (v. Preisz, 1925; Zdansky, 1925; Hauduroy, 1924b; Burnet, 1925). Frequently lysis of bacteria without any swelling whatsoever has been reported (Manninger, 1926b; Burnet, 1925; v. Preisz, 1925). It was not until Bronfenbrenner studied this point by direct cinematographic records of lysing cultures that satisfactory descriptions of the phenomenon were made (Bronfenbrenner, Muckenfuss and Hetler, 1927). In these pictures swollen forms which rapidly disappear leaving a minute trace of amorphous debris are clearly shown. As a rule the debris likewise disappears and the area previously occupied by the cell contains nothing but a few refractile granules. Bayne-Jones and Sandholtzer (1933) have improved on Bronfenbrenner's technic and have followed in great detail the changes occurring in cultures of B. coli and B. megatherium during phage action. They con-

clude that in the case of B. coli swelling usually precedes lysis; the terminal stage of lysis is explosive and a large amount of cellular debris remains after lysis is complete. B. megatherium undergoes lysis without any preliminary enlargement, the terminal stage of lysis is much slower than that observed with B. coli, and again lysis leaves a residue of irregularly shaped granules.

In substantiating the moving picture record Bronfenbrenner demonstrated swelling of bacterial cells due to phage action by measuring the increase in viscosity of the phage-bacterial mixture. Using Kunitz's empirical formula relating viscosity and volume of solute he showed that the volume occupied by the swollen cells was from 6 to 12 times that occupied by the same number of normal bacteria.

B. The effect of phage on bacterial growth. d'Herelle (1926) has recorded the stimulation of bacterial growth by phage. Bronfenbrenner, Muckenfuss and Hetler (1927) reported similar growth stimulation directly observable in his moving picture films. Later Bronfenbrenner and Hetler (1928) and Hetler and Bronfenbrenner (1932) showed that the density of bacterial growth on solid media is increased under drops of phage if special precautions are taken to prevent lysis. In a recent paper by Doubly and Bronfenbrenner (1933) the increased growth of broth cultures of staphylococcus and of B. coli in the presence of phage is recorded.

In the studies of Krueger and Northrop (1930) on the mechanism of the phage-bacterium reaction no bacterial growth stimulation due to phage was observed. These observations on a particular strain of staphylococcus have been repeated many times by Elberg, Baldwin and Scribner, in the writer's laboratory (unpublished experiments), with the same results; practically identical results were obtained by Andrewes and Elford (1932) in the case of B. coli growing in the presence of phage. Lin (1934) repeated the work of Krueger and Northrop with a culture of Shiga dysentery and Shiga phage, measuring the growth rate by means of a Pulfrich photometer; he found no evidence of growth stimulation. Similarly in the work of Eaton (1931) previously referred to, the staphylococcal culture used did not grow faster in the presence of phage. Bordet (1931) also observed no stimulation of growth rate in the presence of phage. Hallauer (1933) could detect no change in bacterial respiration nor in a number of metabolic activities studied during the production of phage in an actively growing culture, nor was Wholfeil (1928) able to find significant differences in the respiratory activity of B. coli cultures growing in the presence and in the absence of phage.

C. Hydrolytic cleavage of bacterial proteins during lysis. Numerous

attempts have been made to determine whether or not the cell proteins are hydrolysed during the lytic process. Several investigators failed to find evidence that such cleavage occurs (Ionesco-Mihaiesti, 1924; Weiss and Arnold, 1924; Zdansky, 1925) although it must be admitted, as Bronfenbrenner (1928) has pointed out, that the negative results may be due to the fact that the culture media used contained relatively high concentrations of protein hydrolytic products as a growth substrate obscuring possible small differences occasioned by hydrolytic cleavage of cell proteins.

Bronfenbrenner, Muckenfuss and Hetler (1927) and Hetler and Bronfenbrenner (1928) using synthetic media free from proteins for growth and lysis of cultures observed a definite increase in the concentration of protein hydrolytic products. Similarly Otto and Sukiennikowa (1923) are of the opinion that protein hydrolysis occurs during lysis. In more recent work by Bayne-Jones and Sandholzer (1933) these authors criticize Bronfenbrenner's hypothesis as to the mechanism of lysis; they feel that the antigenic properties of the lysate indicate that no extensive hydrolysis of bacterial protein is produced by phage. Also in their work they found that the residue after lysis is particulate and consequently could not be the product of hydrolytic cleavage.

D. Development of resistance to phage and concomitant changes in bacterial properties. d'Herelle has described the development of bacterial growth (secondary cultures) in phage lysates when the cleared broth cultures are kept in the incubator for some length of time (d'Herelle, 1920 a, 1921 b). These he ascribed to the operation of natural selection, some bacilli showing the ability to acquire resistance to bacteriophage. He contends that certain cultures may display a natural resistance to some races of phage although they may be completely lysed by other races. Again a different culture may be so completely susceptible to phage action that all cells are lysed before resistant forms have an opportunity to develop (Asheshov, 1925 a). When a phage resistant growth appears he conceives of it as an immunization of bacteria against phage action. d'Herelle's interpretation of the experimental facts has not been accepted universally although development of bacterial growth in filtered lysates has been amply confirmed (Dimtza, 1926–27; Grumbach and Dimtza, 1927; Kauffmann, 1926; Sonnenschein, 1925–26).

When a secondary culture is transferred to bouillon there is developed a growth in which resistant bacteria and phage co-exist—a mixed culture (Bordet and Ciuca, 1920 b). The resistance developed against a

particular phage does not imply resistance to other phages (Applemans and Wagemans, 1922; Bail, 1923–24; Bruynoghe, 1924–25; Gratia, 1923; Hoder, 1924; Matsumoto, 1923, 1923–24). It is therefore a commonly observed fact that the development of resistance may be specific within narrow limits for a given phage. Further, secondary cultures do not represent a permanent resistant state for the capacity to resist phage action may be readily lost if the bacteria are repeatedly transferred to new media or if phage is eliminated from the mixture in some way (as by neutralization with anti-serum) (Bordet and Ciuca, 1921 c; Breinl and Hoder, 1925; Eliava and Pozerski, 1921 a; Saldanha, 1918–24; Bronfenbrenner and Korb, 1925–26; Bronfenbrenner, Muckenfuss and Korb, 1926). At times and under special conditions resistance against phage action may be maintained over long periods even when the bacteria are cultured in the absence of phage (Bronfenbrenner and Korb, 1925–26; Bronfenbrenner, Muckenfuss and Korb, 1926).

Flu (1923 a) has reported the destruction of phage by resistant bacteria which have developed in the filtrate. d'Herelle (1926) has confirmed this observation.

From what has been said already it is clear that the development of resistance is not a simple phenomenon and the specific immunization concept of d'Herelle is made more difficult to uphold by additional experimental facts; for example, phage resistant forms have been obtained from normal cultures by inducing dissociation with heat or chemical treatment (Gratia, 1921 a; Bordet and Ciuca, 1921 a; Hoder and Suzuki, 1927). Schwartzman (1926) has shown in the case of B. coli that the adjustment of pH can bring about development of resistant forms in the absence of phage. Kleineberger (1929 b), in following variants produced over a long period of time from a single strain of colon bacillus, noted the greatest difference in phage susceptibility and phage resistance. Sanjiva (1932) records alterations in the phage resistance of cholera vibrios brought about by adjustment of pH. Even without the use of any chemical or heat treatment it is possible to isolate from normal cultures certain types which prove resistant to phage (Arkwright, 1921, 1924; Gratia, 1921 b, 1923).

Besides the development of resistance to phage the bacterial growth developing in the presence of phage often exhibits a wide variety of altered characters. It is likely that some of these alterations were noted even before d'Herelle had completed his earlier investigations; for example, Gildemeister (1916–17) described atypical colonies which he called "flättenformen" developing in cultures of B. typhosus and B.

coli. In 1921 Kuttner observed irregular colonies of atypical form and since that time many workers have recorded similar observations. In a general way bacteria which have developed resistance show the greatest range of variation in colonial types. d'Herelle (1921 a) states that in his experience mixed colonies developing from cultures displaying high resistance and containing highly active phage are almost always small, viscous and slowly growing. Bordet and Ciuca (1920 b) have described remarkable variation in the colony forms developed during acquisition of resistance to phage in a B. coli culture. The type and extent of variation is not limited to any single bacterial species. In the case of the staphylococcus Gratia and Jaumain (1921) have shown that the growth types may vary tremendously and that this variation may render identification difficult since it involves factors such as pigment production, ordinarily employed as criteria for classification. A large number of publications have appeared in which careful studies of the colony variations are described (d'Herelle, 1920 a; Arkwright, 1924; Gjørup, 1925; Dutton, 1926; Manninger, 1926 a, 1926 b). Since the time of these earlier publications, interesting records of the most bizarre changes in bacteria have accumulated. For example, Kobayashi (1927) produced a form growing like a streptococcus through the interaction of a Shiga phage on B. dysenteriae Shiga. Kleineberger (1929 b) followed the spontaneous production of variants from a single B. coli strain together with variation in phage sensitivity over a period of two years. Eight distinct strains differing widely in susceptibility to phage were obtained. Grumbach (1930) has studied the colony types in streptococcus cultures and reports the occurrence of 80 per cent of variant colony types most of which were associated with phage. Fursenko (1930) has described in detail the production of colony variants from B. typhosus, B. dysenteriae, B. coli communis, and staphylococcus albus. Swieszkowski (1931) reports the dissociating colonial types produced by the action of phage on B. coli; four different forms are recorded. Burnet (1929 b) investigated smooth-rough variation in relation to phage and found that the type of resistant colony produced is related to definite characteristics of the phage used. For example, phages acting on S type organisms produce R resistants; and races acting on R cultures provoke the appearance of S forms. In a later communication Burnet (1929 c) notes that phage-resistant strains which show no such change of phase may develop in lysates. It should be mentioned that the growth of these variants in broth is likewise changed. Typically very dense agglutinated masses which are not dissociated by shaking will collect on the bottom of the tube (d'Herelle, 1921 a).

d'Herelle has pointed out that a frequent morphological change occurring concomitantly with the development of resistance to phage is the production of cocco-bacillary forms or even true coccus forms derived from typical rods. A number of investigators have studied the microscopic changes produced and have recorded interesting manifestations of this tendency (d'Herelle, 1920 a, 1921 b; Burnet, 1925; Kauffmann, 1926; Manninger, 1926 b). Among the more recent studies may be mentioned those of Majer (1927) who worked with B. coli; Kobayashi (1927) who studied a streptococcal variant of Shiga dysenteriae organisms; Simie (1929) who described a morphologically altered form of B. typhosus; and Fursenko (1930) who recorded the production of every conceivable type of microscopic form from the colon-typhoid-dysentery group and from staphylococcus albus. Mackie (1933) attributes the natural variability of the cholera vibrio to phage action. An additional and rather frequent external manifestation of acquired resistance to phage is the development of a capsule. Slime-forming variants not only resist the phage which occasioned their production but are equally resistant to all phages (Kimura, 1925). If the organisms are de-capsulated by the action of bile (Sonnenschein, 1926) susceptibility to lysis is regained.

d'Herelle (1926) has reported extensive changes in cell metabolism as evidenced by altered fermentative characteristics of dysentery bacilli which have become phage resistant. Other workers have recorded similar observations for a variety of phages and bacterial strains (Fejgin, 1923; Gratia, 1922; Hadley, 1924; Hauduroy, 1922 a; Majer, 1927; Simie, 1929; Grumbach, 1930).

Another modification accompanying the acquisition of phage resistance is the loss of agglutinability with specific antisera (Janzen and Wolff, 1923; Gratia, 1922; d'Herelle, 1921 a). These changes may be sufficient to render difficult the identification of a recently isolated strain (d'Herelle, 1920 a; Hoder and Suzuki, 1927; Matsumoto, 1923). Considerable differences in viability may occur; as a rule the resistant forms are less susceptible to lethal environmental changes (Fejgin, 1923; d'Herelle, 1921 a; d'Herelle and Hauduroy, 1925).

In 1920 Bordet and Ciuca (1920 b) found that a phage resistant B. coli strain showed increased virulence for laboratory animals and Gratia and Dath (1924), Davison (1921), Dutton (1926), Kauffmann (1926) have confirmed their findings. Occasionally it happens that virulence may be diminished instead of increased (Blair, 1924; Bronfenbrenner, Muckenfuss and Korb, 1926; Fejgin, 1924). Kendrick (1933) produced variants of S. suipestifer by the action of phage on

virulent S and non-virulent R types. Variants from the S forms in general lost virulence. Sonnenschein (1929 a, 1929 b) observed the production of a variant from B. typhii grown in the presence of phage which produced hemolysis on sheep blood agar plates. He was able to induce the variation in old and recently isolated strains and produced similar variants from B. coli and B. suipestifer. Kleineberger (1930 a) has developed hemolytic variants by phage action on Kruse-Sonne dysentery bacillus, colon bacillus and non-hemolytic streptococcal strains.

Changes in Gram characteristics have been recorded by d'Herelle (1926), Kobayashi (1927) and others. Kleineberger (1929 a) studied the rate of production of variants by means of phage action. From his experiments he concludes that the faster the rate of bacterial reproduction and phage production the more rapid is the formation of mutants.

In more recent publications d'Herelle has given careful consideration to the problem of bacterial mutants. He distinguishes between transformations occurring in the course of life cycles and the abrupt alterations produced in the course of mutation. The latter process he defines as occurring in a disorderly fashion, each unit of character being modified without relationship to other characters. In the case of the cholera vibrio living symbiotically with phage, mutations are common and it is d'Herelle's opinion that many bacteria which have been described as species are actually mutants. d'Herelle and Rakieten (1934) have studied ten mutants derived from one strain of S. enteritidis by phage action; they infer that they actually created a new group of bacteria. Their work stresses the process of natural evolution among bacteria; it is concluded that all the species of Salmonella, Eberthella, Escherichia and Shigella have a common ancestor from which they originated by phage action. In their opinion pathogenicity is governed by phage mutation and the sensitivity of mutants to serum bacteriolysis is a consequence of phage action.

In 1921 Izar reported the appearance of bacterial growth in *filtered* phage lysates. As a rule cocco–bacilli appeared after periods varying from 48 hours to 11 days. Tomaselli (1923) has made similar observations and he concludes from his experiments that resistant ultra-bacteria may pass through filter candles; this phenomenon was not restricted to any one bacterial species. d'Herelle (1926) has had comparable experience with a wide range or organisms. Hauduroy (1924a, 1924b) attempted to determine the numbers of ultra-bacteria appearing in filtered lysates of the colon-typhoid-dysentery group. Judging from his

results the distribution of these forms is sparse. Pryer (1925) describes a filterable stage of staphylococcus produced by phage action. The secondary growth developing from this phase has the microscopic appearance of the diphtheria bacillus. Fejgin (1926) records the isolation of filterable forms from sea water in the presence of bacteriophage.

Bronfenbrenner and Muckenfuss (1927a) attribute the appearance of secondary growths in phage filtrates to such factors as chance contaminations and to altered filtration conditions permitting the passage of organisms. Careful efforts on the part of Burnet (1926) failed to demonstrate the production of filterable forms of B. dysenteriae, nor could Frobisher (1928) produce ultra-bacteria by phage action. He ascribes the secondary growths occasionally occurring as due to defective filters or technical errors. It seems to the writer that the interpretation of experiments purporting to demonstrate the passage of filterable forms through kieselguhr candles should be circumspect in the extreme since it is known that the retention by filter candles of particles in suspension is governed not only by pore size but by a variety of factors such as the pressure used, the composition and pH of the suspending medium, etc. Conceivably a few intact bacterial cells which have resisted phage action may occasionally pass through the filter and account for the positive results recorded. An analogous situation has obtained in connection with the reported filterable forms of B. tuberculosis. In the experiments of Walker and Sweeney (1934) the tubercle bacillus was found quite frequently in filtrates and it was not necessary to assume that a special filterable form of the organism was developed. Using the ultrafilters of Krueger and Ritter (1929), Lewis, Ruckman and James (1935) attempted to recover filterable forms of M. tuberculosis from cultures maintained under conditions said to favor their production. The filtrates did not produce tuberculosis lesions nor a positive tuberculin reaction when injected into guinea pigs; no microscopic forms were found in the centrifuged filtrates and there was a total absence of growth on Sweeney's egg-glycerol medium. In more recent work Kendall and Walker (1933) have claimed that lysed cultures contain invisible, unstainable and uncultivable filterable bacteria which can be made to produce normal bacteria by growth on "K" medium, exposure to bile, or contact with anti-phage. Carpenter and Long (1933) attempted unsuccessfully to demonstrate filterable forms in phage lysates using Kendall's technic. Other investigators have reported similar negative results so that at the present time it is impossible to state definitely that there is clear-cut evidence for the existence of filterable forms produced

by phage action. In view of the many errors to which filter candle technic is susceptible positive results must be accepted cautiously.

THE QUESTION OF AN INDEPENDENT PHAGE METABOLISM. d'Herelle has advanced indirect evidence for the possession of an autonomous metabolism by bacteriophage. It is his contention (1924) that the production of an antigenically distinct lytic substance from a bacterial culture involves transformation of the bacterial substrate into phage and that this implies a separate metabolism for phage. At first glance the early observation of Wollman (1921) seems to offer supporting evidence. Wollman grew dysentery organisms within collodion sacs which were impermeable to dysentery phage placed around the outside of the sacs. During the period of bacterial growth within the sacs the concentration of phage outside increased 10 to 15 times; this, Wollman interpreted as development of phage by utilization of diffusible bacillary products. Asheshov in unreported experiments (d'Herelle, 1926, p. 130) confirmed Wollman's findings. However, Wollman (1925) himself later questioned his original results. Even if the experimental results were entirely correct they do not constitute proof, in the writer's opinion, of an autonomous phage metabolism. One might, with equal facility, assume the secretion of some phage precursor into the medium by the bacteria. This material conceivably having a smaller particle size than phage, could traverse the collodion membrane and might then be transformed into phage by the phage itself. Analogous phenomena among enzyme systems are not uncommon. Furthermore, the greatest significance must be accorded the classical proof presented by Stanley (1935) in connection with his crystalline tobacco mosaic virus that at least one virus is an autocatalytic protein capable of being propagated in contact with living susceptible cells. This bears rather indirectly on the nature of phage and then only by analogy but it clearly defines one instance of what may very well prove to be a general case. With this work in mind it is not at all unreasonable to entertain the idea that an inanimate agent, (phage, for instance) destructive to certain cells may be reproduced by those same cells.

Although Jötten (1922) reported the successful production of phage on dead bacteria no one has confirmed his finding (Otto, Munter and Winkler, 1922; Seiffert, 1923–24a; Burnet, 1925; Bronfenbrenner and Muckenfuss, 1927b) and it is generally conceded that phage formation is intimately linked with bacterial reproduction. Consequently such indirect experiments may be said to have failed in supplying a clear-cut demonstration that phage metabolizes independently of bacteria.

The most direct means of detecting autonomous metabolic activity on the part of phage is through the measurement of gas exchange in lysates by means of microrespirometers. Bronfenbrenner experimented along these lines some ten years ago (Bronfenbrenner, 1924–25, 1926; Bronfenbrenner and Reichert, 1926–27) with entirely negative results. Bachmann and Wohlfeil (1927) likewise could not detect any evidence of phage respiration; confirmatory negative experiments were later reported by Wohlfeil (1928) and Walthard (1931).

The observations on phage metabolism mentioned above were made with bacteria-free phage suspensions. Similar measurements of gaseous interchange have been made using bacterial cultures growing in the presence of homologous phage. Bronfenbrenner and Reichert (unpublished experiments, 1925) found no evidence of CO_2 production attributable to phage. Wohlfeil (1928) obtained similar results. Eaton (1931) has since presented experimental evidence which indicates that phage on or in combination with bacteria respires. Eaton's data show that a culture lysed by phage and containing negligible numbers of living bacteria continues to give off CO_2 and to take up O_2 for several hours after the completion of lysis. It is possible that cellular fragments may account for these findings, for some cell fragments, at any rate, are known to possess respiratory activity a considerable length of time after fragmentation has occurred. Schüler (1935) working with phage purified by Schlesinger's method found no evidence of independent metabolism in the presence of either living or dead bacteria.

Besides the attempts to measure phage respiration in the absence and in the presence of growing susceptible cells, experiments have been undertaken to determine the independent metabolism of phage in terms of its reducing power. The rate of reduction shown by cultures of susceptible bacterial cells is independent of the concentration of lytic principle added (Schwartzmann, 1926–27; Kauffmann, 1925–26; Gozony and Suranyi, 1925; Fejgin, 1924). To date then, no convincing experimental evidence has accumulated indicating that phage possesses an independent metabolism.

THE ANTIGENICITY OF PHAGE. In the course of investigations of phage Bordet and Ciuca (1921b) demonstrated that the repeated injection of phage into animals causes the appearance of anti-phage substances in the animal serum. This has been confirmed repeatedly (Asheshov, 1925b; Osumi, 1924; Watanabe, 1923a; Sonnenschein, 1925). Phages display a considerable variation in their capacity to evoke the appearance of anti-phage substances in the serum (Wagemans,

1923a; Otto, Munter and Winkler, 1922) which latter in addition to its neutralizing capacity also contains agglutinins, opsonins, precipitins and complement fixing antibodies for the organism used in preparing the lysate (Bruynoghe and Dubois, 1927; Bail, 1921; da Costa Cruz, 1926; Hauduroy, 1922b; Marcuse, 1925–26). Burnet (1933a) has recently reported the specific agglutination of a large particle phage with anti-phage serum. Phage inactivated by heat, formalin or exposure to light in the presence of a suitable dye is still agglutinable. Schlesinger (1934b) and Bertarelli (1934) have observed this same phenomenon. When purified phage is used as the antigen the corresponding serum contains anti-phage but does not react with the bacteria themselves (Arnold and Weiss, 1925; Bossa, 1932; Kligler and Olitzki, 1934). Larkum (1933), injected staphylococcal phage purified by the method of Krueger and Tamada into rabbits and has claimed the production of staphylococcus anti-toxin comparable in potency to that produced by the crude lysate. Larkum contends that phage itself is the antigen concerned in the production of staphylococcal antitoxin and that phage is apparently a toxoid.

It is interesting that ordinary anti-bacterial serum prepared without the use of phage in any way, possesses the property of inhibiting bacteriophagy (Bail, 1921; da Costa Cruz, 1926, Hauduroy, 1922b; Marcuse, 1925–26). Hauduroy (1922b) has shown that this effect is not on the phage but seems to be due to action on the bacteria rendering them less susceptible to lysis.

There seems to be no doubt that phage is an independent antigenic substance. In the first place, as already noted, purified phage will cause the production of anti-phage without evoking anti-bacterial substances in the serum. Further evidence is found in the case of phages acting on closely related species; the antigenic specificity of the filtrate is constant for each phage and is not dependent upon the bacterial substrate used in propagating the phage (Bruynoghe, 1924–25; Bruynoghe and Dubois, 1927; Seiffert, 1923–24b; Wagemans, 1923b). Also it is noteworthy that if antiserum prepared with an active phage filtrate is subjected to absorption with homologous bacteria the residual serum retains its capacity to neutralize phage (da Costa Cruz, 1926; v. Preisz, 1925; Weiss and Arnold, 1924). Jungeblut and Schultz (1929) in a study of the sensitizing properties of phage lysates found that specific contractions of the uterine strips of guinea pigs actively sensitized with phage-lysed dysentery organisms or colon bacilli, lysed by the same phage, occurred when the tissues were exposed to the homologous phage lysate. These reactions did not occur when the same phage propagated on het-

erologous organisms was used in the test. Animals sensitized with phage-free antigens did not give reactions with corresponding phage lysates. These investigators suggest that a new and immunologically distinct antigenic complex is produced during lysis of bacteria with phage.

Phage absorbed by homologous bacteria causes the latter to be specifically agglutinated by the corresponding phage serum. In one instance this reaction was obtained with formalin inactivated phage and the corresponding organism (Burnet, 1933d).

To summarize, it has been shown that a crude phage lysate injected parenterally into an animal causes the appearance in the serum of antibodies active against both the phage and the bacterial components of the lysate. The anti-phage substances specifically neutralize phage and flocculate the phage particles, while those developed against the bacteria consist of agglutinins, precipitins, opsonins, and complement fixing antibodies. Purified phage, on the other hand, does not evoke the production of anti-bacterial antibodies but causes agglutinins and neutralizing substances active against phage to appear in the serum.

THE ACTION OF ANTI-PHAGE SERUM ON PHAGE. In 1921 Bordet and Ciuca (1921b) reported that neutralization of phage with anti-serum involved destruction of the phage. A number of investigators have disproven this contention by demonstrating that dissociation of the phage anti-phage complex with recovery of the two constituents in active form can occur (d'Herelle and Eliava, 1921; Seiffert, 1923–24b; Weiss, 1927a). More recent evidence along the same lines is contained in the work of Andrewes and Elford (1933b) who note dissociation of the phage-anti-phage combination following simple dilution and in the work of Burnet (1933e) who has reported the detection of a thermolabile phage soluble specific substance which blocks the phage anti-phage reaction and will reverse the reaction to some extent if present in sufficient concentration. Kligler and Olitzki (1932) working with protein-free suspensions were unable to detect reversibility of the phage-antibody combination.

There has been considerable diversity of opinion as to the nature of the inactivation noted with anti-phage. Several authors have shown that complement-free serum retains its neutralizing powers (Asheshov et al., 1930; Seiffert, 1923–24b; Wagemans, 1923b; Watanabe, 1923a; Schultz et al., 1928–29). A few, particularly Osumi (1924) and Kligler and Olitzki (1932), are of the opinion that complement plays a part in the reaction.

Andrewes (1932) found that homologous phage taken up by living

colon bacilli is protected from the neutralizing effect of anti-serum at low temperatures. The reports that complement does not play a part in the neutralization of phage by specific antiserum and the positive observations on the reversal of the phage antibody neutralization have led several writers to conclude that the neutralization phenomenon is analogous to toxin neutralization by antitoxin. To gather further evidence along these lines, efforts were made to determine whether phage and anti-phage react according to the law of multiple proportions. Positive results have been reported by Wagemans (1923b), Arnold and Weiss (1924) and Schultz et al. (1929, 1931). According to the careful investigations of Andrewes and Elford (1933a) the reaction does not follow the law of multiple proportions. These workers found that neutralization is demonstrable only in the presence of a large excess of antiserum and that a given quantity of antiserum neutralizes a certain percentage of phage regardless of the original phage concentration.

THE ORIGIN OF PHAGE. Theories concerning the nature of phage have in general considered two possibilities; first, that phage is a microorganism foreign to the bacterium and propagating at its expense, and second, that phage is a non-living entity derived in some way from the bacterium and capable of causing the production of more of itself when brought into contact with susceptible organisms. Hypotheses embodying either of these concepts together with varying subsidiary corollaries have been advanced in considerable numbers and the arguments centering around them constitute a formidable fraction of phage literature. It seems somewhat beside the point to analyze these theories in any detailed fashion since at the present time the truth of any particular one cannot be established to the exclusion of all others due primarily to the lack of unanimity regarding essential primary facts about phage.

If it were possible regularly and at will to generate phage from normal bacterial cells the theory of the living nature of phage would fall. Several investigators have recorded successful attempts to derive phage in this way by a variety of experimental procedures (Blair, 1924; Bordet and Ciuca, 1920a, 1920b; Burgers and Bachman, 1923–24; Gratia 1921c; Jötten, 1922; Kuttner, 1920–21; Otto, Munter and Winkler, 1922; Lisbonne, Boulet and Carrere, 1922; Manoliu, 1925; Pico, 1922–1923; Putter and Vallen, 1923; Fukuda, 1928; LeMar and Myers, 1935), but it must be admitted that their results are open to criticism. In the first place lysogenic bacteria or phage-carrying strains are of much more common occurrence than was previously supposed. For example, Burnet (1932) surveyed 130 stock Salmonella strains using three indi-

cator or highly phage-susceptible bacterial types to test their phage forming capacity. Ninety-three of the 130 strains regularly produced phage capable of lysing one or more of the indicator strains. Similarly Smith and Jordan (1931) found that a large number of diphtheria strains were lysogenic toward a sensitive indicator strain of the same species. Not only do these bacterial types continually produce phage in the course of their growth but it has been shown that in the case of certain spore formers the lysogenic tendency survives any degree of heat which leaves the spore viable (Cowles, 1931; Den Dooren de Jongh, 1931). Clearly then if the experimental results are to have significance, all attempts to derive phage from bacteria must be prefaced by a careful demonstration proving that the bacterial strain in question is not lysogenic.

In addition the widespread distribution of phage in nature complicates experimental procedures, for phage has been shown to be widely distributed in a variety of animal tissues (Flu, 1923b; d'Herelle, 1921d, 1922b; Hoder and Suzuki, 1926; Pico, 1924; Wolff, 1925) and experiments in which bacteria are treated with products of animal origin for the induction of phage formation must be looked at askance. Also it is possible to pick up phage by contamination quite readily through dust or droplet conveyance (Colvin, 1932a, 1932b) or during the frequent filtrations through candles (Beard, 1931; Putter and Vallen, 1923) so often employed.

To be accorded serious consideration a method for the generation of phage de novo should involve a rigid technic excluding not only all possibility of using lysogenic strains but the chance of phage contamination during experimental manipulation as well. Furthermore, the technic should be regularly effective on all bacterial types known to be naturally phage-susceptible.

At the moment then there exists no clear-cut experimental evidence that phage can be derived from phage-free bacterial strains and the main argument which might be advanced to support the "non-living" theory is consequently inadequately backed. The possibility of obtaining direct information by separating phage in pure form from the heterogenous solutions in which it occurs is being exploited in several laboratories; if pure phage is prepared its nature probably can be determined quite readily. Until then the protagonists of the "non-living" theory can muster the following facts to bolster their position: 1, phage does not possess the power of assimilation; it lacks an independent metabolism; 2, phage is not adaptable.

The foundation of d'Herelle's thesis that phage is a living ultramicroscopic virus rests upon the capacity of phage to assimilate, i.e., to change bacterial substance into phage substance and upon its ability to adapt itself. Since neither of these characteristics can be attributed to phage in the light of present knowledge the burden of proof would appear to rest with the adherents of the "living" theory. The latter have emphasized the absurdity of a conception which involves the multiplication of an inanimate substance, lethal for certain cells, upon a substrate consisting of these same living cells. There are no longer valid philosophical objections to considering phage in this light for Stanley (1935) has isolated a crystalline protein of constant composition which possesses all the infective properties of tobacco mosaic virus. Consequently for the first time proof is available that in one case at least, a disease virus is an autocatalytic protein requiring the presence of living susceptible cells for its propagation and a consideration of phage in the same terms is certainly within the realm of reason.

It is quite likely that phage either is a protein or, more certainly perhaps, that its activity is in some manner dependent upon a protein structure in phage. This is suggested by the very high temperature coefficient for heat inactivation, μ, the critical thermal increment, being 101,000 (Krueger, 1932a); critical thermal increments of this magnitude are uniquely characteristic of protein heat denaturation in general. Schlesinger's elementary analyses of purified phage, (1934a) are likewise indicative of a protein structure. Phage apparently is a complete antigen and antigens typically, although not always, are proteins.

As has been pointed out in several of the above sections phage possesses many of the properties of an enzyme. However, extension of the enzyme analogy through a more detailed analysis of the characteristics shared by phage and enzymes is of decidedly dubious value in offering proof that phage actually is an enzyme. Even though they shared a great number of common attributes the very valid objection could always be raised that similar series of parallel data would hold for enzymes and living cells without any implication as to the identity of the two. A specific example is to be found in the relationships obtaining for heat inactivation of enzymes and heat killing of bacteria. Both processes exhibit a remarkable rise in rate for a small temperature increment; Chick (1910) reported a Q_{10}° of 110 for the killing of typhoid bacteria in hot water and similar figures have been recorded for the heat inactivation of a number of enzymes. As Anson and Mirsky (1931)

have observed protein denaturation alone displays such high temperature coefficients and it could be deduced quite rationally that both the heat killing of typhoid bacteria and the heat inactivation of enzymes involve protein denaturation as a significant reaction. One would predict such a similarity since the maintenance of life processes by the typhoid bacillus depends upon the enzymes it produces. It would be a fallacy, however, to conclude that the sharing of a property, even a unique property, indicates the identity of typhoid bacilli and enzymes. For just this reason the experimental demonstration that phage and enzymes possess identical properties cannot lead to conclusive proof that phage is an enzyme.

Despite the many difficulties which are always encountered in purifying substances of high biological activity when only minute amounts are present in complex solutions there can be no doubt that the current trend points to the successful purification of phage within a relatively short time. The problem of the origin of phage remains unsolved although to the writer the known facts seem to favor a bacterial origin.

UNICITY AND MULTIPLICITY OF PHAGE: THE CLASSIFICATION OF PHAGES. According to d'Herelle phage is a single substance capable of adapting itself to various bacterial types and all phage variants belong to a single species as evidenced by the fact that many pure phage races possess multiple virulence and by the experimental adaptation of phage to bacterial species previously not susceptible. Several authors have reported such adaptations (Applemans 1923; Ionesco-Mihaiesti, 1924; McKinley, 1922–23; Wollman and Wollman, 1927) while others have failed to elicit the phenomenon (Bail, 1923–24; Epstein and Fejgin, 1926; Marshall, 1925; Matsumoto, 1924; Beard, 1931). Naturally the successful experiments, as has been pointed out by Bronfenbrenner, are open to two criticisms; first, that the phage originally may possess a latent activity for the bacterium to which it is adapted, the adaptation consisting in merely uncovering a latent lytic capacity, and second, that the pure phage may be actually a mixture of phages. This latter concept is supported by the demonstrable fact that the original activity toward one bacterium may be lost—as if by dilution—during adaptation toward another bacterium.

There seems to be no reasonable doubt that there is not one phage but many phages, each possessing individual characteristics which under set conditions are fairly constant and which vary widely between phages. Among these characteristics the most important are: *a*, strict serologic specificity independent of the bacterial substrate (Bail and Watanabe,

1922; Bruynoghe and Applemans, 1922; Bruynoghe, 1924–25; Gratia, 1923; Seiffert, 1923–24b; Wagemans, 1923b); *b*, resistance to physical and chemical agents (Bronfenbrenner and Korb, 1925b; Brutsaert, 1924; Bruynoghe, 1924–25; Gratia 1923; Osumi, 1924; Wagemans, 1923b); *c*, plaque characteristics (Bruynoghe, 1924–25; Hoder, 1924; Matsumoto, 1923–24; Wagemans, 1923b); *d*, particle size (Elford and Andrewes, 1932); *e*, the capacity to produce bacterial forms resistant to certain groups of phages.

Bail (1923–24), first clearly showed the possibility of differentiating phages on the basis of plaque size, cross resistance tests as mentioned in *e* above, and serologic specificity. Among phages lysing a single bacterial strain the greatest differences between plaques produced under standard conditions may be noted and the plaque size is relatively constant for a given pure phage. Elford and Andrewes (1932) have shown that there exists an inverse relationship between plaque size and particle size of a phage; the smaller the phage the more readily it can diffuse through agar and consequently the larger the plaques.

As has been mentioned in a previous section, a secondary bacterial growth usually develops after completion of lysis in a mixture of phage and bacteria and this secondary culture is resistant to the phage employed. When such a resistant form is tested for susceptibility to various other phages it will be lysed by some and not by others. In Bail's classification all phages which fail to lyse the same resistant bacterium belong to an identical resistance group together with the phage which provokes the development of the resistant form. Employing the three criteria of plaque size, resistance tests, and serologic specificity Bail classified a large group of anti-dysentery phages. His results were confirmed and extended by Burnet and McKie (1933), Burnet (1933b) and by Morison (1932). Similar data have been collected on the properties of various phages acting on a wide range of bacterial types by Burnet and McKie (1929), Asheshov et al. (1930), Bronfenbrenner and Korb (1925b), Bruynoghe (1924–25), and others. Probably the most significant studies along these lines are those of Burnet and McKie (1933), and Burnet (1933b), in view of the fact that the particle size of many of their phages was accurately determined by Elford and Andrewes (1932). About fifty phages active on dysentery and coli strains were classified by the three criteria already mentioned. The results show that the phages of a given serologic group are always uniform with respect to plaque size and major resistance group. Phages of a certain particle size may belong to any of the major resistance groups

and each of the latter includes phages of various particle sizes. Evidently serologic grouping is the most satisfactory for species differentiation while the resistance groups seem to give only indirect indications of differences between phages. The sharp limits between phage groups defined by serologic tests find support in biochemical evidence recorded by Burnet (1933c). He noted that the capacity of phages to develop on susceptible bacterial substrates in calcium-free media, their resistance to photodynamic inactivation by certain dyes and their resistance to urea solutions varied with the serologic group to which the phages belonged.

It is Burnet's opinion, in which he is joined by many other workers, that the term "bacteriophage" includes species just as definite as bacterial species and like them labile in certain of their properties.

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